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(54) Title: NUCLEIC ACIDS ENCODING POLYPEPTIDES HAVING ABSIDIA LIPASE ACTIVITY

(57) Abstract

The present invention relates to isolated nucleic acid sequences encoding polypeptides having *Absidia* lipase activity. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing the polypeptides. The invention further relates to compositions comprising the polypeptides.

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NUCLEIC ACIDS ENCODING POLYPEPTIDES HAVING ABSIDIA LIPASE ACTIVITY

Background of the Invention

Field of the Invention

The present invention relates to isolated nucleic acid sequences encoding polypeptides having *Absidia* lipase activity. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as recombinant methods for producing the polypeptides.

Description of the Related Art

Detergents formulated with lipolytic enzymes are known to have improved properties for removing fatty stains. For example, LIPOLASE™ (Novo Nordisk A/S, Bagsvaerd, Denmark), a microbial lipase obtained from the fungus *Thermomyces lanuginosus* (also called *Humicola lanuginosa*), has been introduced into many commercial brands of detergent.

Other microbial lipases from *Pseudomonas cepacia* (U.S. Patent No. 4,876,024), *Streptomycetes* (WO 94/14940), and *Gongronella butleri* strain NRRL 3521 (U.S. Patent No. 3,634,195, the strain was previously named *Absidia butleri*, see K.H. Domsch *et al.*, *Compendium of Soil Fungi*, Academic Press 1980, p. 381) have also been suggested for use in detergents.

U.S. Patent No. 3,634,195 describes the production of lipase from *Absidia cylindrospora* var. *rhizomorpha* NRRL 2815 and *Absidia blakesleeana* NRRL 1305. Koritala *et al.* (1987, *Journal of the American Oil Chemists Society* 64: 509-513) disclose that soybean oil was partially hydrolyzed when incubated with *Absidia coerula* NRRL 5926 and *Absidia ramosa* NRRL 1309. Satyanarayana (1981, *Current Science* 50: 680-682) discloses the secretion of lipase by a strain of *Absidia corymbifera*. Aisaka *et al.* (1979, *Agricultural Biological Chemistry* 43: 2125-2129) describe the formation of a lipoprotein lipase from *Absidia hyalospora* strain KY 303 (now classified as *Absidia blakesleeana*).

Many detergents are alkaline in solution (*e.g.*, around pH 10) and contain a builder to bind Ca^{++} ions. There is a need for new lipolytic enzymes with high activity at high pH in the absence of Ca^{++}. Lipases of the genus *Absidia* possess these characteristics, and therefore, are highly desirable for use in detergent compositions. Heretofore, however, there has been no means of producing these enzymes recombinantly.

It is an object of the present invention to provide for recombinant production of these valuable enzymes.

Summary of the Invention

The present invention relates to isolated nucleic acid sequences encoding polypeptides having lipase activity selected from the group consisting of:

(a) a nucleic acid sequence which encodes a polypeptide endogenous to an *Absidia* strain with an amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10;

(b) a nucleic acid sequence endogenous to an *Absidia* strain which is capable of hybridizing under medium stringency conditions with (i) the nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9 or (ii) any of their complementary strands;

(c) a nucleic acid sequence which is capable of hybridizing under medium stringency conditions with (i) the nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9 or (ii) any of their complementary strands;

(d) a nucleic acid sequence encoding a polypeptide having lipase activity with an amino acid sequence which has at least 65% identity with the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10;

(e) an allelic form of (a), (b), (c), or (d); and

(f) a fragment of (a), (b), (c), (d), or (e).

The present invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as recombinant methods for producing the polypeptides.

Brief Description of the Figures

Figure 1 shows an agarose gel purification of lipase-specific PCR products from *Absidia griseola* and *Absidia griseola* var. *iguchii* genomic DNA. Lane 1: *Hin*dIII-digested lambda DNA and *Hae*III-digested øX17RF-DNA size standards; lane 2: *Absidia griseola* PCR product; and lane 3: *Absidia griseola* var. *iguchii* PCR product. Both PCR products appear to be approximately 870 bp in size.

Figure 2 shows an autoradiogram from Southern hybridization analysis of genomic DNA digests from several *Absidia* species probed with a radiolabeled lipase gene segment from *Absidia griseola*. The sizes of *Hin*dIII-digested lambda DNA and *Hae*III-digested øX17RF-DNA size standards are indicated on the right side of the autoradiogram. Lanes 1-3: *Absidia sporophora-variabilis* DNA (*Eco*RI plus *Asp*718I, *Asp*718I and *Eco*RI, respectively); lanes 4-6: *Absidia corymbifera* DNA (*Eco*RI plus *Asp*718I, *Asp*718I and *Eco*RI, respectively); lanes 7-9: *Absidia blakesleeana* DNA (*Eco*RI plus *Asp*718I, *Asp*718I and *Eco*RI, respectively); lanes 10-12: *Absidia*

griseola var. *iguchii* DNA (*Eco*RI plus *Asp*718I, *Asp*718I and *Eco*RI, respectively); and lanes 13-15: *Absidia griseola* DNA (*Eco*RI plus *Asp*718I, *Asp*718I and *Eco*RI, respectively).

Figure 3 shows the DNA sequence and deduced amino acid sequence of *Absidia griseola* var. *iguchii* lipase (SEQ ID NOS. 1 and 2, respectively). Introns are marked by a solid line. Regions corresponding to previously determined peptide sequences are underlined (---).

Figure 4 shows the DNA sequence and deduced amino acid sequence of *Absidia blakesleeana* lipase (SEQ ID NOS. 3 and 4, respectively). Introns are marked by a solid line. Regions corresponding to previously determined peptide sequences are underlined (---).

Figure 5 shows the DNA sequence and deduced amino acid sequence of *Absidia corymbifera* lipase (SEQ ID NOS. 5 and 6, respectively). Introns are marked by a solid line. Regions corresponding to previously determined peptide sequences are underlined (---).

Figure 6 shows the DNA sequence and deduced amino acid sequence of *Absidia sporophora-variabilis* lipase (SEQ ID NOS. 7 and 8, respectively). Introns are marked by a solid line.

Figure 7 shows the DNA sequence and deduced amino acid sequence of *Absidia reflexa* lipase (SEQ ID NOS. 9 and 10, respectively).

Figure 8 shows a comparison of the amino acid sequence homology among *Absidia* lipases compared to *Rhizomucor miehei* lipase (SEQ ID NO:15) and *Humicola lanuginosa* (SEQ ID NO:16). Identical residues are boxed.

Figure 9 shows a restriction map of pBANe6.

Figure 10 shows a restriction map of pKB2.

Figure 11 shows a restriction map of pRamB19.

Detailed Description of the Invention

Nucleic Acid Sequences

In a first embodiment, the present invention relates to isolated nucleic acid sequences which encode polypeptides having lipase activity with an amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10. In a specific embodiment, the nucleic acid sequences are set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, and SEQ ID NO:9. The nucleic acid sequences of the present invention also encompass nucleic acid sequences which encode a polypeptide having the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10, but differ from SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, respectively, by virtue of the degeneracy of the genetic code. The present invention also relates to subsequences of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, which encode a fragment of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10, respectively, and retain lipase

activity. In a preferred embodiment, the nucleic acid sequences of the present invention are the nucleic acid sequences contained in plasmids pZL-NL1, pZL-NL61, pZL-NL95, and pZL-NL124, which are contained in *Escherichia coli* NRRL B-21520, NRRL B-21521, NRRL B-21522, and NRRL B-21523, respectively.

The term "isolated nucleic acid sequence" as used herein refers to a nucleic acid sequence which is essentially free of other nucleic acid sequences, *e.g.*, at least about 20% pure, preferably at least about 40% pure, more preferably at least about 60% pure, even more preferably at least about 80% pure, and most preferably at least about 90% pure as determined by agarose electrophoresis. For example, an isolated nucleic acid sequence can be obtained by standard cloning procedures used in genetic engineering to relocate the nucleic acid sequence from its natural location to a different site where it will be reproduced. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a host cell where multiple copies or clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

The term "lipase" is defined herein as a lipolytic enzyme classified under the Enzyme Classification number E.C. 3.1.1.- (Carboxylic Ester Hydrolases) in accordance with the Recommendations (1992) of the International Union of Biochemistry and Molecular Biology (IUBMB). Lipolytic enzymes thus exhibit hydrolytic activity towards at least one of the types of ester bonds mentioned in the context of E.C. 3.1.1, for example, ester bonds present in mono-, di- and triglycerides, phospholipids (all classes), thioesters, cholesterol esters, wax-esters, cutin, suberin, synthetic esters, etc. As an example, the lipolytic enzymes of the present invention may have activity towards triglycerides (lipase activity, E.C. 3.1.1.3), *e.g.*, 1,3-positionally specific lipase activity.

In a second embodiment, the present invention relates to isolated nucleic acid sequences encoding polypeptides with lipase activity which are capable of hybridizing under high, medium, or low stringency conditions with an oligonucleotide probe which hybridizes under the same conditions with the nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, its complementary strand or a subsequence thereof (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). Hybridization indicates that the analogous nucleic acid sequence hybridizes to the oligonucleotide probe corresponding to the polypeptide encoding part of the nucleic acid sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, under low to high stringency conditions (for example, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and either 50, 35 or 25% formamide for high, medium and low stringencies, respectively), following standard Southern

blotting procedures. In a preferred embodiment, the nucleic acid sequences are capable of hybridizing under medium stringency conditions and most preferably under high stringency conditions. In another preferred embodiment, the nucleic acid sequences are capable of hybridizing with the nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, or its complementary strand.

SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, as well as SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10, or subsequences thereof may be used to design an oligonucleotide probe to isolate homologous genes encoding lipases from other strains of different genera or species according to methods well known in the art. Thus, a genomic or cDNA library prepared from such other organisms may be screened for DNA which hybridizes with such probes following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, preferably at least 25, and more preferably at least 40 nucleotides in length. Longer probes, preferably no more than 1200 nucleotides in length, can also be used. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P, ^{3}H, biotin, or avidin). A PCR reaction using the degenerate probes mentioned herein and genomic DNA or first-strand cDNA from an *Absidia* strain can also yield an *Absidia* lipase-specific product which can then be used as a probe to clone the corresponding genomic or cDNA.

Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify clones or DNA which are homologous with SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, the carrier material is used in a Southern blot in which the carrier material is finally washed three times for 30 minutes each using 2X SSC, 0.2% SDS at preferably not higher than 40°C, more preferably not higher than 45°C, more preferably not higher than 50°C, more preferably not higher than 55°C, even more preferably not higher than 60°C, especially not higher than 65°C. Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using X-ray film.

The present invention also relates to isolated nucleic acid sequences which have a degree of identity to the nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9 of at least about 65%, preferably about 70%, preferably about 75%, preferably about 80%, more preferably about 85%, even more preferably about 90%, most preferably about 95%, and even most preferably about 97%, which encode an active polypeptide. The degree of identity between two nucleic acid sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman and Wunsch,

1970, *Journal of Molecular Biology* 48: 443-453). For purposes of determining the degree of identity between two nucleic acid sequences for the present invention, the Clustal method (DNASTAR, Inc., Madison, WI) is used with an identity table, a gap penalty of 10, and a gap length of 10.

In a third embodiment, the present invention relates to isolated nucleic acid sequences encoding polypeptides with lipase activity which have an amino acid sequence with a degree of identity to the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10 of at least about 65%, preferably about 70%, preferably about 75%, preferably about 80%, more preferably about 85%, even more preferably about 90%, most preferably about 95%, and even most preferably about 97%, which qualitatively retain the activity of the polypeptides (hereinafter "homologous polypeptides"). In a preferred embodiment, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10. The degree of identity between two or more amino acid sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman and Wunsch, 1970, *Journal of Molecular Biology* 48: 443-453). For purposes of determining the degree of identity between two amino acid sequences for the present invention, the Clustal method (DNASTAR, Inc., Madison, WI) is used with an identity table, a gap penalty of 10, and a gap length of 10.

The amino acid sequences of the homologous polypeptides encoded by the nucleic acid sequences of the present invention may differ from the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10 by an insertion or deletion of one or more amino acid residues and/or the substitution of one or more amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions which do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine and histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine and valine), aromatic amino acids (such as phenylalanine, tryptophan and tyrosine), and small amino acids (such as glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, *e.g.*, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most

commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse.

The isolated nucleic acid sequences of the present invention which are capable of hybridizing with an oligonucleotide probe which hybridizes with the nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, its complementary strand or a subsequence thereof, may be obtained from microorganisms of any genus, for example, from a bacterial or fungal source, but preferably from a fungal cell, and more preferably from a filamentous fungal cell or a yeast cell. For purposes of the present invention, the term "obtained from" (or endogenous to) as used herein in connection with a given source shall mean that the polypeptide is produced by the source or by a cell in which a gene from the source has been inserted. Preferred sources for homologous genes are strains of the genus *Absidia* and species thereof available in public depositories. Furthermore, homologous genes may be identified and obtained from other sources including microorganisms isolated from nature (*e.g.*, soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art. The nucleic acid sequence may then be derived by similarly screening a cDNA library of another microorganism. Particularly preferred strains are filamentous fungus strains, such as an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* strain or yeast strains, such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* strain.

In a preferred embodiment, a nucleic acid sequence of the present invention is obtained from a strain of the genus *Absidia*, as described in M.A.A. Schipper, Persoonia, Vol. 14, Part 2, pp. 133-148 (1990), such as a strain of *Absidia griseola*, *Absidia sporophora-variabilis*, *Absidia griseola* var. *iguchii*, *Absidia corymbifera*, or *Absidia blakesleeana*. In an even more preferred embodiment, the nucleic acid sequence is obtained from *Absidia blakesleeana* NN100826 (NRRL 1304), *e.g.*, the nucleic acid sequence set forth in SEQ ID NO:3; *Absidia corymbifera* NN100062 (IFO 8084), *e.g.*, the nucleic acid sequence set forth in SEQ ID NO:5; *Absidia griseola* NN000987 (ATCC 20430); *Absidia griseola* var. *iguchii* NN000591 (ATCC 20431), *e.g.*, the nucleic acid sequence set forth in SEQ ID NO:1; *Absidia sporophora-variabilis* NN102427 (ATCC 36019), *e.g.*, the nucleic acid sequence set forth in SEQ ID NO:7; and *Absidia reflexa* NN102427 (ATCC 44896), *e.g.*, the nucleic acid sequence set forth in SEQ ID NO:9.

Within the genus *Absidia*, the following subgenera, groups, species and strains are preferred. Variants and mutants thereof capable of producing lipolytic enzyme are also encompassed. It is noted that a number of previously recognized species names were reclassified by Schipper, *Op. cit.*,

and for convenience the previously used names of some strains are also listed below where multiple numbers in the same box indicate multiple deposits of the same strain.

Subgenus, group	Species name	Previous species name	Inventors' strain No.	Deposit number(s)
Subgenus *Mycocladus*	*A. blakesleeana*	*A. blakesleeana*	NN100826	NRRL 1304, ATCC 10148a, CBS 100.28, CMI 111736
		A. blakesleeana	NN102406	CBS 100.36
		A. blakesleeana	NN102407	CBS 102.36, NRRL 2696
		A. blakesleeana	NN102408	CBA 420.70
		A. blakesleeana	NN102413	NRRL 1305
		A. griseola	NN000987	ATCC 20430
		A. griseola	NN102403	CBS 519.71, ATCC 22618, IFO 9472
		A. griseola var. *iguchii*	NN000591	ATCC 20431
		A. hyalospora	NN102432	CBS 173.67, NRRL 2916
	A. blakesleeana var. *atrospora*	*A. atrospora*	NN102423	CBS 518.71, ATCC 22617, IFO 9471
	A. corymbifera	*A. corymbifera*	NN100060	CBS 100.31, IFO 4009, NRRL 2982
		A. corymbifera	NN100062	IFO 8084
		A. corymbifera	NN102404	CBS 102.48
		A. corymbifera	NN102405	CBS 582.65, ATCC 22574, NRRL 1309
		A. hesseltinii	NN102426	CBS 958.68, ATCC 24263

Subenus *Absidia*, Group B	*A. cylindrospora* var. *rhizomorpha*	-	NN102422	CBS 154.63, NRRL 2815*A. pseudocylindrospo ra*- NN102434ATCC 24169, CBS 100.62, NRRL 2770
-	*A. reflexa*	-	NN102424	ATCC 44896, IFO 5874
-	*A. sporophora-variabilis*	-	NN102427	ATCC 36019

The above-mentioned strains are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

Once a nucleic acid sequence has been detected with the probe(s) described above, the sequence may be isolated or cloned by utilizing techniques which are well known to those of ordinary skill in the art (see, *e.g.*, Sambrook *et al.*, *supra*). The known techniques used to isolate or clone a nucleic acid sequence include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleic sequences of the present invention from such genomic DNA can be effected, *e.g.*, by using the well known polymerase chain reaction (PCR). See, for example, Innis *et al.*, 1990, *A Guide to Methods and Application*, Academic Press, New York. The nucleic acid sequence may be cloned from a strain of *Absidia* producing the polypeptide, or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleic acid sequence.

Modification of the nucleic acid sequence encoding the polypeptide may be necessary for the synthesis of polypeptides substantially similar to the polypeptide. The term "substantially similar" to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source. For example, it may be of interest to synthesize variants of the polypeptide where the variants differ in specific activity, thermostability, oxidative stability, pH optimum, or the like using, for example, site-directed mutagenesis. The analogous sequence may be constructed on the basis of the nucleic acid sequence presented as the polypeptide encoding region of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ

ID NO:7, or SEQ ID NO:9, a sub-sequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the polypeptide encoded by the nucleic acid sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, *e.g.*, Ford *et al.*, 1991, *Protein Expression and Purification* 2: 95-107.

It will be apparent to those skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the polypeptide encoded by the isolated nucleic acid sequence of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, *e.g.*, Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for lipase activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (see, *e.g.*, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *Journal of Molecular Biology* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Letters* 309: 59-64).

Polypeptides encoded by the nucleic acid sequences of the present invention also include fused polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleic acid sequence (or a portion thereof) encoding another polypeptide to a nucleic acid sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include, ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

The polypeptides having lipolytic activity encoded by the nucleic acid sequences of this invention are characterized by having a high activity at alkaline pH (about pH 9-10), even in the absence of free Ca^{++}.

More specifically, these polypeptides have optimum lipolytic activity at about pH 9 or higher (have a higher activity at pH 9 than at pH 8) when tested in the absence of free Ca^{++}.

Certain preferred nucleic acid sequences encode polypeptides having lipase activity when tested at pH 9 without free Ca^{++}. Such lipolytic enzymes can be obtained from strains of *Absidia* subgenus *Mycocladus*, *e.g.*, the species and strains listed above.

Another group of preferred nucleic acid sequences encode polypeptides having a higher lipolytic activity at pH 10 than pH 9 in the absence of Ca^{++}. Such a nucleic acid sequence can be obtained from *Absidia reflexa* NN102427 (ATCC 44896).

A further group of preferred nucleic acid sequences encodes polypeptides retaining more than 90% residual activity after 30 minutes incubation at pH 10, 45°C. Such a sequence can be obtained from a strain of *Absidia sporophora-variabilis*, *e.g.*, *Absidia sporophora-variabilis* NN102427 (ATCC 36019).

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a nucleic acid sequence of the present invention operably linked to one or more control sequences capable of directing the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

"Nucleic acid construct" is defined herein as a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. The term nucleic acid construct may be synonymous with the term expression cassette when the nucleic acid construct contains all the control sequences required for expression of a coding sequence of the present invention. The term "coding sequence" as defined herein is a sequence which is transcribed into mRNA and translated into a polypeptide of the present invention when placed under the control of the appropriate control sequences. The boundaries of the coding sequence are generally determined by a translation start codon ATG at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, DNA, cDNA, and recombinant nucleic acid sequences.

An isolated nucleic acid sequence encoding a polypeptide of the present invention may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the nucleic acid sequence encoding a polypeptide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying nucleic acid sequences utilizing cloning methods are well known in the art.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for expression of the coding sequence of the nucleic acid sequence. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, a polyadenylation sequence, a propeptide sequence, a promoter, a signal sequence, and a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

The control sequence may be an appropriate promoter sequence, a nucleic acid sequence which is recognized by a host cell for expression of the nucleic acid sequence. The promoter sequence contains transcription control sequences which mediate the expression of the polypeptide. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention, especially in a bacterial host cell, are the promoters obtained from the *E. coli lac* operon, the *Streptomyces coelicolor* agarase gene (*dagA*), the *Bacillus subtilis* levansucrase gene (*sacB*), the *Bacillus licheniformis* alpha-amylase gene (*amyL*), the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), the *Bacillus licheniformis* penicillinase gene (*penP*), the *Bacillus subtilis xylA* and *xylB* genes, and the prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; and in Sambrook *et al.*, 1989, *supra*.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Fusarium oxysporum* trypsin-like protease (as described in U.S. Patent No. 4,288,627, which is incorporated herein by reference), and hybrids thereof. Particularly preferred promoters for use in filamentous fungal host cells are the TAKA amylase, NA2-tpi (a hybrid of the promoters from the genes encoding *Aspergillus niger* neutral a-amylase and *Aspergillus oryzae* triose phosphate isomerase), and *glaA* promoters.

In a yeast host, useful promoters are obtained from the *Saccharomyces cerevisiae* enolase (ENO-1) gene, the *Saccharomyces cerevisiae* galactokinase gene (GAL1), the *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase genes (ADH2/GAP), and the *Saccharomyces cerevisiae* 3-phosphoglycerate kinase gene. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-488. In a mammalian host cell, useful promoters include viral promoters such as those from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus, and bovine papilloma virus (BPV).

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used in the present invention.

Preferred terminators for filamentous fungal host cells are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes encoding *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), or *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*. Terminator sequences are well known in the art for mammalian host cells.

The control sequence may also be a suitable leader sequence, a nontranslated region of a mRNA which is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence which is functional in the host cell of choice may be used in the present invention.

Preferred leaders for filamentous fungal host cells are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase and *Aspergillus oryzae* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the *Saccharomyces cerevisiae* enolase (ENO-1) gene, the *Saccharomyces cerevisiae* 3-phosphoglycerate kinase gene, the *Saccharomyces cerevisiae* alpha-factor, and the *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase genes (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence which is operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the host cell of choice may be used in the present invention.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, and *Aspergillus niger* alpha-amylase.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Molecular Cellular Biology* 15: 5983-5990. Polyadenylation sequences are well known in the art for mammalian host cells.

The control sequence may also be a signal peptide coding region, which codes for an amino acid sequence linked to the amino terminus of the polypeptide which can direct the expressed polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleic acid

sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which is foreign to that portion of the coding sequence which encodes the secreted polypeptide. The foreign signal peptide coding region may be required where the coding sequence does not normally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to obtain enhanced secretion of the lipase relative to the natural signal peptide coding region normally associated with the coding sequence. The signal peptide coding region may be obtained from a glucoamylase or an amylase gene from an *Aspergillus* species, a lipase or proteinase gene from a *Rhizomucor* species, the gene for the alpha-factor from *Saccharomyces cerevisiae*, an amylase or a protease gene from a *Bacillus* species, or the calf preprochymosin gene. However, any signal peptide coding region capable of directing the expressed lipase into the secretory pathway of a host cell of choice may be used in the present invention.

An effective signal peptide coding region for bacterial host cells is the signal peptide coding region obtained from the maltogenic amylase gene from *Bacillus* NCIB 11837, the *Bacillus stearothermophilus* alpha-amylase gene, the *Bacillus licheniformis* subtilisin gene, the *Bacillus licheniformis* beta-lactamase gene, the *Bacillus stearothermophilus* neutral proteases genes (*nprT*, *nprS*, *nprM*), and the *Bacillus subtilis prsA* gene. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

An effective signal peptide coding region for filamentous fungal host cells is the signal peptide coding region obtained from *Aspergillus oryzae* TAKA amylase gene, *Aspergillus niger* neutral amylase gene, the *Rhizomucor miehei* aspartic proteinase gene, the *Humicola lanuginosa* cellulase gene, or the *Rhizomucor miehei* lipase gene.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding regions are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding region, which codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the *Bacillus subtilis* alkaline protease gene (*aprE*), the *Bacillus subtilis* neutral protease gene (*nprT*), the *Saccharomyces cerevisiae* alpha-factor gene, or the *Myceliophthora thermophilum* laccase gene (WO 95/33836).

The nucleic acid constructs of the present invention may also comprise one or more nucleic acid sequences which encode one or more factors that are advantageous in the expression of the polypeptide, *e.g.*, an activator (*e.g.*, a trans-acting factor), a chaperone, and a processing protease. Any factor that is functional in the host cell of choice may be used in the present invention. The nucleic acids encoding one or more of these factors are not necessarily in tandem with the nucleic acid sequence encoding the polypeptide.

An activator is a protein which activates transcription of a nucleic acid sequence encoding a polypeptide (Kudla *et al.*, 1990, *EMBO Journal* 9: 1355-1364; Jarai and Buxton, 1994, *Current Genetics* 26: 2238-244; Verdier, 1990, *Yeast* 6: 271-297). The nucleic acid sequence encoding an activator may be obtained from the genes encoding *Bacillus stearothermophilus* NprA (*nprA*), *Saccharomyces cerevisiae* heme activator protein 1 (*hap1*), *Saccharomyces cerevisiae* galactose metabolizing protein 4 (*gal4*), and *Aspergillus nidulans* ammonia regulation protein (*areA*). For further examples, see Verdier, 1990, *supra* and MacKenzie *et al.*, 1993, *Journal of General Microbiology* 139: 2295-2307.

A chaperone is a protein which assists another polypeptide in folding properly (Hartl *et al.*, 1994, *TIBS* 19: 20-25; Bergeron *et al.*, 1994, *TIBS* 19: 124-128; Demolder *et al.*, 1994, *Journal of Biotechnology* 32: 179-189; Craig, 1993, *Science* 260: 1902-1903; Gething and Sambrook, 1992, *Nature* 355: 33-45; Puig and Gilbert, 1994, *Journal of Biological Chemistry* 269: 7764-7771; Wang and Tsou, 1993, *The FASEB Journal* 7: 1515-11157; Robinson *et al.*, 1994, *Bio/Technology* 1: 381-384). The nucleic acid sequence encoding a chaperone may be obtained from the genes encoding *Bacillus subtilis* GroE proteins, *Aspergillus oryzae* protein disulphide isomerase, *Saccharomyces cerevisiae* calnexin, *Saccharomyces cerevisiae* BiP/GRP78, and *Saccharomyces cerevisiae* Hsp70. For further examples, see Gething and Sambrook, 1992, *supra*, and Hartl *et al.*, 1994, *supra*.

A processing protease is a protease that cleaves a propeptide to generate a mature biochemically active polypeptide (Enderlin and Ogrydziak, 1994, *Yeast* 10: 67-79; Fuller *et al.*, 1989, *Proceedings of the National Academy of Sciences USA* 86: 1434-1438; Julius *et al.*, 1984, *Cell* 37: 1075-1089; Julius *et al.*, 1983, *Cell* 32: 839-852). The nucleic acid sequence encoding a processing protease may be obtained from the genes *Saccharomyces cerevisiae* dipeptidylaminopeptidase, *Saccharomyces cerevisiae* Kex2, and *Yarrowia lipolytica* dibasic processing endoprotease (*xpr6*).

It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems would include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and the *Aspergillus oryzae* glucoamylase promoter may be used as

regulatory sequences. Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene which is amplified in the presence of methotrexate, and the metallothionein genes which are amplified with heavy metals. In these cases, the nucleic acid sequence encoding the polypeptide would be placed in tandem with the regulatory sequence.

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a nucleic acid sequence of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleic acid and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the polypeptide at such sites. Alternatively, the nucleic acid sequence of the present invention may be expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression, and possibly secretion.

The recombinant expression vector may be any vector (*e.g.*, a plasmid or virus) which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleic acid sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The vector system may be a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon.

The vectors of the present invention may be integrated into the host cell genome when introduced into a host cell. For integration, the vector may rely on the nucleic acid sequence encoding the polypeptide or any other element of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the host cell. The additional nucleic acid sequences enable the vector to be integrated into the host cell genome at a precise location(s) in the chromosome(s). To increase the likelihood of

integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleic acid sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination. These nucleic acid sequences may be any sequence that is homologous with a target sequence in the genome of the host cell, and, furthermore, may be non-encoding or encoding sequences.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAMß1 permitting replication in *Bacillus*. Examples of origin of replications for use in a yeast host cell are the 2 micron origin of replication, the combination of CEN6 and ARS4, and the combination of CEN3 and ARS1. The origin of replication may be one having a mutation which makes its functioning temperature-sensitive in the host cell (see, *e.g.*, Ehrlich, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1433). In a specific embodiment, the expression vector may be pZL-NL1, pZL-NL61, pZL-NL95, or pZL-NL124.

The vectors of the present invention preferably contain one or more selectable markers which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. A frequently used mammalian marker is the dihydrofolate reductase gene. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. A selectable marker for use in a filamentous fungal host cell may be selected from the group including, but not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hygB* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenyltransferase), *trpC* (anthranilate synthase), and glufosinate resistance markers, as well as equivalents from other species. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* markers of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* marker of *Streptomyces hygroscopicus*. Furthermore, selection may be accomplished by co-transformation, *e.g.*, as described in WO 91/17243, where the selectable marker is on a separate vector.

More than one copy of a nucleic acid sequence encoding a polypeptide of the present invention may be inserted into the host cell to amplify expression of the nucleic acid sequence. Stable amplification of the nucleic acid sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome using methods well known in the art and selecting for transformants.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

Host Cells

The present invention also relates to recombinant host cells, comprising a nucleic acid sequence of the invention, which are advantageously used in the recombinant production of the polypeptides. The term "host cell" encompasses any progeny of a parent cell which is not identical to the parent cell due to mutations that occur during replication.

The cell is preferably transformed with a vector comprising a nucleic acid sequence of the invention followed by integration of the vector into the host chromosome. "Transformation" means introducing a vector comprising a nucleic acid sequence of the present invention into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleic acid sequence is more likely to be stably maintained in the cell. Integration of the vector into the host chromosome occurs homologous or non-homologous recombination as described above.

The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source. The host cell may be a unicellular microorganism, *e.g.*, a prokaryote, or a non-unicellular microorganism, *e.g.*, a eukaryote. Useful unicellular cells are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, *e.g.*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, *e.g.*, *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp. In a preferred embodiment, the bacterial host cell is a *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus stearothermophilus* or *Bacillus subtilis* cell. The transformation of a bacterial host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), by using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), by electroporation (see, *e.g.*, Shigekawa and Dower,

1988, *Biotechniques* 6: 742-751), or by conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278).

The host cell may be a eukaryote, such as a mammalian cell, an insect cell, a plant cell or a fungal cell. Useful mammalian cells include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, or any number of other immortalized cell lines available, *e.g.*, from the American Type Culture Collection.

In a preferred embodiment, the host cell is a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*). Representative groups of Ascomycota include, *e.g.*, *Neurospora*, *Eupenicillium* (=*Penicillium*), *Emericella* (=*Aspergillus*), *Eurotium* (=*Aspergillus*), and the true yeasts listed above. Examples of Basidiomycota include mushrooms, rusts, and smuts. Representative groups of Chytridiomycota include, *e.g.*, *Allomyces*, *Blastocladiella*, *Coelomomyces*, and aquatic fungi. Representative groups of Oomycota include, *e.g.*, Saprolegniomycetous aquatic fungi (water molds) such as *Achlya*. Examples of mitosporic fungi include *Aspergillus*, *Penicillium*, *Candida*, and *Alternaria*. Representative groups of Zygomycota include, *e.g.*, *Rhizopus* and *Mucor*.

In a preferred embodiment, the fungal host cell is a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). The ascosporogenous yeasts are divided into the families Spermophthoraceae and Saccharomycetaceae. The latter is comprised of four subfamilies, Schizosaccharomycoideae (*e.g.*, genus *Schizosaccharomyces*), Nadsonioideae, Lipomycoideae, and Saccharomycoideae (*e.g.*, genera *Pichia*, *Kluyveromyces* and *Saccharomyces*). The basidiosporogenous yeasts include the genera *Leucosporidim*, *Rhodosporidium*, *Sporidiobolus*, *Filobasidium*, and *Filobasidiella*. Yeast belonging to the Fungi Imperfecti are divided into two families, Sporobolomycetaceae (*e.g.*, genera *Sorobolomyces* and *Bullera*) and Cryptococcaceae (*e.g.*, genus *Candida*). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, Soc. App. Bacteriol. Symposium Series No. 9, 1980. The biology of yeast and manipulation of yeast genetics are well known in the art (see, *e.g.*, *Biochemistry and Genetics of Yeast*, Bacil, M., Horecker, B.J., and Stopani, A.O.M., editors, 2nd edition, 1987; *The Yeasts*, Rose, A.H., and Harrison, J.S., editors, 2nd edition, 1987; and *The Molecular Biology of the Yeast Saccharomyces*, Strathern *et al.*, editors, 1981).

In a more preferred embodiment, the yeast host cell is a cell of a species of *Candida*, *Kluyveromyces*, *Saccharomyces*, *Schizosaccharomyces*, *Pichia*, or *Yarrowia*.

In a most preferred embodiment, the yeast host cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* cell. In another most preferred embodiment, the yeast host cell is a *Kluyveromyces lactis* cell. In another most preferred embodiment, the yeast host cell is a *Yarrowia lipolytica* cell.

In a preferred embodiment, the fungal host cell is a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are characterized by a vegetative mycelium composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative. In a more preferred embodiment, the filamentous fungal host cell is a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, and *Trichoderma*.

In an even more preferred embodiment, the filamentous fungal host cell is an *Aspergillus* cell. In another even more preferred embodiment, the filamentous fungal host cell is an *Acremonium* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Fusarium* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Humicola* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Mucor* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Myceliophthora* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Neurospora* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Penicillium* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Thielavia* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Tolypocladium* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Trichoderma* cell.

In a most preferred embodiment, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus oryzae* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium graminearum*, *Fusarium oxysporum*, *Fusarium sambucinum* or *Fusarium sulphureum* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Humicola insolens* or *Humicola lanuginosa* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Mucor miehei* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Myceliophthora thermophilum* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Neurospora crassa* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Penicillium purpurogenum* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Thielavia terrestris* cell. In

another most preferred embodiment, the *Trichoderma* cell is a *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei* or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81:1470-1474. A suitable method of transforming *Fusarium* species is described by Malardier *et al.*, 1989, *Gene* 78: 147-156 or in copending US Serial No. 08/269,449, incorporated herein by reference. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology*, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920. Mammalian cells may be transformed by direct uptake using the calcium phosphate precipitation method of Graham and Van der Eb (1978, *Virology* 52: 546).

Methods of Production

The present invention also relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a host cell under conditions conducive to expression of the polypeptide; and (b) recovering the polypeptide.

In these methods, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, *e.g.*, references for bacteria and yeast; Bennett, J.W. and LaSure, L., editors, *More Gene Manipulations in Fungi*, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it is recovered from cell lysates.

The polypeptides may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide. The production of lipase activity can be determined by any method known in the art. In one method, one Lipase Unit (LU) is the amount of

enzyme which liberates 1.0 µmol of titratable fatty acid per minute with tributyrin as substrate and gum arabic as an emulsifier at 30°C, pH 7.0 (phosphate buffer). Lipolytic enzyme activity in the absence of free Ca^{++} in the range pH 7-10 is tested with a substrate emulsion of olive oil: 2% PVA solution (1:3) at 40°C for 10 minutes, at a specified pH. At the end of the reaction, the reaction mixture is extracted by chloroform: methanol (1:1) at acidic conditions, and the fatty acid released during the reaction is measured by TLC-FID analysis (Iatroscan). One OPID unit (OPIDU) is taken as the release of 1.0 µmole of fatty acid per minute. In each test, 10 mM EDTA is used together with 200 mM of buffer (Tris-HCl buffer at pH 7 and 8, diethanol amine buffer at pH 8, 9 and 10).

The resulting polypeptide may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The recovered polypeptide may then be further purified by a variety of chromatographic procedures, *e.g.*, ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like.

The polypeptides of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing (IEF), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

Uses

The recombinant polypeptides enoded by the nucleic acid sequences of the invention may be used in conventional applications of lipolytic enzyme, particularly at a high pH, *e.g.*, in laundry and dishwashing detergents, institutional and industrial cleaning and leather processing.

The lipolytic enzymes of the invention can also be used for interesterification, for total hydrolysis of fats and oils, and in optical isomer resolution process.

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

Examples

Example 1: Genomic DNA Extraction

Absidia griseola NN000987 (ATCC 20430), *Absidia sporophora-variabilis* NN102427 (ATCC 36019), *Absidia griseola* var. *iguchii* NN000591 (ATCC 20431), *Absidia corymbifera* NN100062 (IFO 8084), and *Absidia blakesleeana* NN100826 (NRRL 1304) were each grown in 25 ml of 0.5% yeast extract-2% glucose (YEG) medium for 24 hours at 32°C and 250 rpm. Mycelia

from each culture were then collected by filtration through Miracloth (Calbiochem, La Jolla, CA) and washed once with 25 ml of 10 mM Tris-0.1 M EDTA (TE) buffer. Excess buffer was drained from the mycelia preparations which were subsequently frozen in liquid nitrogen. The frozen mycelia preparations were ground to a fine powder in an electric coffee grinder, and the powders were each added to a disposable plastic centrifuge tube containing 20 ml of TE buffer and 5 ml of 20% w/v sodium dodecylsulfate (SDS). The mixtures were gently inverted several times to ensure mixing, and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v). Sodium acetate (3 M solution) was added to the extracted samples to a final concentration of 0.3 M followed by 2.5 volumes of ice cold ethanol to precipitate the DNA. The tubes were centrifuged at 15,000 x g for 30 minutes to pellet the DNA. The DNA pellets were allowed to air-dry for 30 minutes before resuspension in 0.5 ml of TE buffer. DNase-free ribonuclease A was added to the resuspended DNA pellets to a concentration of 100 µg/ml and the mixtures were then incubated at 37°C for 30 min. Proteinase K (200 µg/ml) was added and each tube was incubated an additional one hour at 37°C. Finally, each sample was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) before precipitating the DNA with sodium acetate and ethanol. The DNA pellets were dried under vacuum, resuspended in TE buffer, and stored at 4°C.

Example 2: PCR Amplification of *Absidia griseola* NN000987 and *Absidia griseola* var. *iguchii* NN000591 Lipase Gene Segments

Based on the amino acid sequences of the *Absidia griseola* NN000987 lipase and the *Absidia griseola* var. *iguchii* NN000591 lipase as disclosed by Gormsen *et al.* in Patent Application DK 95/00424 (the contents of which are incorporated herein by reference), the oligonucleotide primers shown below were synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer, according to the manufacturer's instructions, to PCR amplify lipase gene fragments from *Absidia griseola* NN000987 and *Absidia griseola* var. *iguchii* NN000591 (Note: R = A or G, I = inosine, Y = T or C, H = A or T or C, and N = A or T or C or G):

1. Forward primer

   Amino acid sequence: GluThrGlulleGlnAlaHisThrPhe (SEQ ID NO:11)

   Oligonucleotide (+ strand): 5'-GARACIGARATHCARGCICAYACITT-3' (SEQ ID NO:12)

2. Reverse primer

   Amino acid sequence: ProProGlyAlaPheGlyPheLeu (SEQ ID NO:13)

   Oligonucleotide (– strand): 5'-ARRAANCCRAAIGCNCCIGGNGG-3' (SEQ ID NO:14)

Amplification reactions (100 ml) were prepared using approximately 1 µg of *Absidia griseola* NN000987 or *Absidia griseola* var. *iguchii* NN000591 genomic DNA as the template. Each reaction contained the following components: 1 µg of genomic DNA, 40 pmol of forward primer, 40 pmol of reverse primer, 200 mM each of dATP, dCTP, dGTP, and dTTP, 1 x *Taq* polymerase buffer

(Perkin-Elmer Corp., Branchburg, NJ), and 5 Units of *Taq* polymerase (Perkin-Elmer Corp., Branchburg, NJ). Sterile mineral oil (100 µl) was layered on top of each reaction mixture, and the reactions were incubated in a Perkin-Elmer Model 480 Thermal Cycler programmed as follows: Cycle 1 - 95°C for 5 minutes, 45°C for 2 minutes, and 67°C for 5 minutes; Cycle 2-30 - 95°C for 2 minutes; 45°C for 2 minutes, and 67°C for 2 minutes; and Soak cycle at 4°C. The reaction products were isolated on a 1% low melting point agarose gel (Sigma Chemical Co., St. Louis, MO) and the major PCR product band from each reaction was excised from the gel and purified using ß-agarase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. The purified PCR products were subsequently cloned into a pCRII vector (Invitrogen, San Diego, CA) and the DNA sequences were determined using *lac* forward and reverse primers (New England BioLabs, Beverly, MA).

Lipase gene segments of approximately 870 bp were amplified from *Absidia griseola* NN000987 and *Absidia griseola* var. *iguchii* NN000591 as shown in Figure 1 with the lipase-specific PCR primers described above. DNA sequence analysis showed that the amplified gene segments encode portions of the corresponding *Absidia* lipase genes. In addition, the DNA sequence data confirmed that these two gene products are probably identical and share regions of homology with the *Rhizomucor miehei* lipase.

Example 3: Hybridization Analysis of Genomic DNA

Total cellular DNA samples prepared from the five *Absidia* strains described in Example 1 were analyzed by Southern hybridization (Maniatis *et al.*, 1982, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York). Approximately 2–5 µg of each DNA sample was digested with *Eco*RI, *Asp*718I or *Eco*RI plus *Asp*718I and fractionated on a 1% agarose gel. The gel was photographed under short wavelength UV light and soaked for 15 minutes in 0.5 M NaOH-1.5 M NaCl followed by 15 minutes in 1.5 M NaCl-1 M Tris-HCl pH 8. DNA in the gel was transferred onto NytranÔ hybridization membrane (Schleicher & Schuell, Keene, NH) by capillary blotting in 20 X SSPE (3 M sodium chloride-0.2 M sodium dibasic phosphate-0.02 M disodium EDTA) according to Davis *et al.* (1980, *Advanced Bacterial Genetics, A Manual for Genetic Engineering*, Cold Spring Harbor Press, Cold Spring Harbor, New York). The DNA was cross-linked onto the membrane using a UV Stratalinker (Stratagene, La Jolla, CA), and the membrane was soaked for 2 hours in the following hybridization buffer at 45°C with gentle agitation: 5 X SSPE, 35% formamide (v/v), 0.3% SDS, and 200 mg/ml denatured and sheared salmon testes DNA. The lipase-specific probe fragment isolated from the *Absidia griseola* NN000987 PCR-clone described in Example 2 was radiolabeled by nick translation (Maniatis *et al.*, 1982, *supra*) with [^{32}P]dCTP (Amersham, Arlington Heights, IL), denatured by adding NaOH to a final concentration of 0.1 M, and added to the hybridization buffer at an activity of approximately 1 x 10^6 cpm per ml of

buffer. The mixture was incubated overnight at 45°C in a shaking water bath. Following incubation, the membranes were washed once in 0.2 X SSPE with 0.1% SDS at 45°C followed by two washes in 0.2 X SSPE (no SDS) at the same temperature. The membranes were allowed to dry on paper towels for 15 minutes, then wrapped in Saran-Wrap™ and exposed to X-ray film overnight at -70°C with intensifying screens (Kodak, Rochester, NY).

Analysis of the total cellular DNA samples from each of the *Absidia* species by Southern blotting under conditions of moderate stringency using the PCR-derived lipase gene segment probe from *Absidia griseola* var. *iguchii* NN000591 demonstrated that the lipase genes of all the *Absidia* species tested cross-hybridized to the probe (Figure 2). All the species tested also showed a single hybridization signal in the *Eco*RI digest of approximately 2.1 kb with the exception of *Absidia sporophora-variabilis* NN102427 which gave a hybridization signal with a 4 kb band. Furthermore, it appeared that all of the species tested contain a single copy of the corresponding lipase gene in their genomes.

Example 4: DNA Libraries and Identification of Lipase Clones

Enriched genomic DNA libraries were constructed in the bacteriophage cloning vector lZipLox (Life Technologies, Gaithersburg, MD). First, total cellular DNA was digested with *Eco*RI and size-fractionated on 1% agarose gels. DNA fragments migrating in the size range corresponding to hybridization signals previously observed on Southern blots described in Example 3 were excised and eluted from the gel using Prep-a-Gene reagents (BioRad Laboratories, Hercules, CA). The approximate sizes of the DNA fragments in these fractions were as follows: 1.9-2.5 kb (*Absidia griseola* var. *iguchii* NN000591), 1.9-2.5 kb (*Absidia blakesleeana* NN100826), 1.9-2.5 kb (*Absidia corymbifera* NN100062) and 2.5-4.3 kb (*Absidia sporophora-variabilis* NN102427). The eluted DNA fragments were ligated with *Eco*RI-cleaved and dephosphorylated λZipLox vector arms (Life Technologies, Gaithersburg, MD), and the ligation mixtures were packaged using commercial packaging extracts (Stratagene, La Jolla, CA). The packaged DNA libraries were plated and amplified in *Escherichia coli* Y1090ZL cells (Life Technologies, Gaithersburg, MD). The titers of recombinant phage in each library ranged from 1.3–5.4 X 10^5 pfu/ml (background titers with no DNA were 1.7–2.0 X 10^4 pfu/ml). Approximately 15,000–30,000 plaques from each unamplified library were screened by plaque-hybridization using the lipase-specific PCR fragment from *Absidia griseola* NN000987 as the probe (Davis *et al.*, 1980, *supra*). Plaques, which gave strong hybridization signals with the probe, were purified twice in *E. coli* Y1090ZL cells and the lipase genes were subsequently excised from the λZipLox vector as pZL1-derivatives (D'Alessio *et al.*, 1992, Focus® 14: 76). The recombinant DNA segments were inserted within the phagemid pZL1 portion of the vector, and the phagemid harboring the cloned insert was recovered in the

autonomously replicating pZL1 using *in vivo* excision by infection of *E. coli* DH10Bzip cells (Life Technologies, Gaithersburg, MD). The lipase clones isolated in this manner were prepared for DNA sequence analysis using a Wizard 373 DNA purification kit (Promega, Madison, WI).

Example 5: DNA Sequence Analysis of Lipase Genes

DNA sequencing of the lipase clones described in Example 4 was performed with an Applied Biosystems Model 373A Automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) on both strands using the primer walking technique with dye-terminator chemistry (Giesecke *et al.*, 1992, *Journal of Virol. Methods* 38: 47-60). Oligonucleotide sequencing primers were synthesized on an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions.

The nucleotide sequences of the genes encoding the *Absidia* lipases are shown in Figures 3-6 (SEQ ID NOS:1, 3, 5, and 7). The assignment of introns within each gene was based on (a) known amino acid sequence data derived from peptide fragments (Boel *et al.*, 1988, *Lipids* 23: 701-706), (b) alignment to the deduced amino acid sequence of *Rhizomucor miehei* lipase (SEQ ID NO:15) (Gurr *et al.*, *In* Kinghorn, J.R. [ed], *Gene Structure in Eukaryotic Microbes*, pp. 93-139, IRL Press, Oxford), and (c) known consensus sequences for introns of filamentous fungi (von Heijne, 1984, *Journal of Molecular Biology* 173: 243-251).

In order to isolate a gene encoding the *Absidia reflexa* lipase, a strain of *Absidia reflexa* NN102427 (ATCC 44896) was grown on optimal medium with jojoba oil as an induction component. A cDNA library was prepared from this strain, and, using the *Absidia corymbifera* gene described above as a probe, a cDNA clone was identified by colony hybridization. The sequence of the *Absidia reflexa* gene is provided in Figure 7 (SEQ ID NO:9). This lipase is approximately 99% identical with the *Absidia sporophora-variabilis* lipase.

Example 6: Comparison of the Lipase Genes from *Absidia* Species

The intron-exon organization among *Absidia* lipase genes is very similar in that all are composed of seven exons and six introns as shown in Table 1. The size of the exons 2 through 6 are strictly conserved. The first exon shows slight variability in size due to some variation in the region encoding the signal peptide and propeptide portions of the enzyme. In addition, the nucleotide sequence homology between corresponding exons is also very high with 85-97% identity. In contrast, the lengths of the introns varied, and with the exception of the *Absidia corymbifera* NN100062 and *Absidia sporophora-variabilis* NN102427 lipase genes, there is little

sequence homology between corresponding introns. The 5' and 3' flanking sequences of the lipase genes also shows sequence divergence.

Table 1. Intron-exon organization of the lipase genes from several *Absidia* species.

Species	Exon (length in bp)							Intron (length in bp)					
	1	2	3	4	5	6	7	1	2	3	4	5	6
A. griseola var. *iguchii*	489	58	97	173	81	79	31	82	64	56	61	64	66
A. blakesleeana	492	58	97	173	81	79	31	75	52	57	60	64	67
A. corymbifera	495	58	97	173	81	79	31	61	62	54	58	53	59
A. sporophora-variabilis	495	58	97	173	81	79	31	61	62	54	57	53	59

Based on the deduced amino acid sequences (SEQ ID NOS:2, 4, 6, 8, and 10), a comparison of the biochemical and biophysical properties of the *Absidia* lipases is presented in Table 2. All four lipases are very similar in that all are synthesized as preproenzymes of 337-338 amino acids, comprising signal peptides of 17-21 amino acids, propeptides of 52-56 amino acids, and mature enzymes of 264-265 amino acids with molecular weights of approximately 29,000 for the non-glycosylated proteins. The calculated isoelectric points vary from 6.10 to 6.94 with the most alkaline being the lipase from *Absidia sporophora-variabilis*. These calculated isoelectric values are much lower than those observed experimentally on IEF gels (see Gormsen *et al.* in Patent Application DK 95/00424) probably as a result that not all charged residues are on the exposed three-dimensional surface of the protein.

Table 2. Predicted biochemical and biophysical properties of from several *Absidia* species.

Species	Preproform	Predicted signal peptide	Predicted propeptide	Mature lipase	Molecular weight	Calculated isoelectric point	Molar extinction coeficient	1 A280 unit (mg/ml)
A. griseola var. *iguchii*	337 aa	18 aa	54 aa	264 aa	29,028	6.45	27,580	1.05
A. blakesleeana	337 aa	18 aa	55 aa	264 aa	28,959	6.64	24,900	1.16
A. corymbifera	338 aa	21 aa	52 aa	265 aa	29,057	6.10	27,460	1.06
A. sporophora-variabilis	338 aa	17 aa	56 aa	265 aa	28,952	6.94	27,460	1.05

The *Absidia* lipases share extensive amino acid sequence homology between each other with 87-96% identity, and limited homology to *Rhizomucor miehei* lipase (SEQ ID NO:15) with 53-55% identity and *Humicola lanuginosa* lipase (SEQ ID NO:16) with 22-24% identity as shown in Table 3 and Figure 8.

Table 3. Amino acid sequence similarity among lipases from *Absidia* species, *R. miehei*, and *H. lanuginosa*

Percent Similarity

		1	2	3	4	5	6		
Percent Divergence	1	-	87.2	87.5	88.1	53.7	22.3	1	*A. blakesleeana*
	2	11.9	-	86.3	87.2	54.8	22.7	2	*A. griseola* var. *iguchii*
	3	11.6	11.9	-	96.4	53.3	23.7	3	*A. corymbifera*
	4	11.0	11.0	3.6	-	53.3	24.1	4	*A. sporophora-variabilis*
	5	41.6	40.5	41.9	41.9	-	23.7	5	*R. miehei*
	6	70.6	70.9	70.0	70.0	68.8	-	6	*Humicola lanuginosa*
		1	2	3	4	5	6		

From a phylogenetic standpoint, the *Absidia* lipases are most closely related to the lipases from other members of the Zygomycete class of fungi. Yeast, bacterial, and mammalian lipases as well as fungal cutinases appear to be very distantly related if at all.

Example 7: Expression of the *Absidia corymbifera* lipase gene

The clone and the nucleotide sequences of the *Absidia corymbifera* lipase gene described above were used for subcloning of the gene and expression in an *Aspergillus* host. PCR was used to subclone the lipase gene (without its own promoter) from the isolated genomic clone NL95A using primers designed from the nucleotide sequences. In order to facilitate the subcloning of the gene fragment into an expression vector designated pBANe6 (Figure 9), *Swa*I and *Pac*I restriction enzyme sites, respectively, at the 5' and 3' end of the gene, were introduced. The vector pBANE6 contained the TAKA promoter, NA2-tpi leader, and AMG terminator as regulatory sequences. The plasmid also contained the *Aspergillus nidulans amdS* gene as a selectable marker for fungal transformations. The following primers were used for PCR amplification:

Forward Primer: 5'-CCCATTTAAATATGCGTTTTTATTCAGTAGTATCAT-3' (SEQ ID NO:17)

Reverse primer: 5'-CTCGGCTTAATTAAAATGGGTTATAAGCAGAGACCAGTG-3' (SEQ ID NO:18)

PCR was performed using *Pwo* polymerase (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's specifications. The PCR amplified product was gel isolated, cut with *Swa*I and *Pac*I enzymes, and gel purified. The purified fragment was ligated to the pBANe6 vector (already cut with *Swa*I and *Pac*I) to yield the plasmid pKB2 (Figure 10) in which transcription of the lipase gene was under the control of the TAKA promoter. The plasmid pKB2 was transformed into *E. coli* DH5 cells. *E. coli* transformants containing the pKB2 plasmid were isolated and plasmid DNA was prepared for transformation and expression in *Aspergillus*.

Protoplasts were prepared from *Aspergillus oryzae* strain BANe3 in which the *amdS* gene of the host strain was deleted. Protoplast preparation and transformation were done as previously described (Christensen *et al., supra*). *Aspergillus oryzae* transformants expressing acetamidase were selected based on their ability to utilize acetamide as the sole nitrogen source. A total of 42 transformants were generated and spore purified twice on selective plates. The spore purified transformants were used for further analysis.

The transformants were screened for lipase expression by cultivation in shake flasks (25 ml medium in 125 ml flasks) using a medium containing 50 g of maltose, 2.0 g of $MgSO_4$-$7H_2O$, 10 g of KH_2PO_4, 2.0 g of K_2SO_4, 2.0 g of citric acid, 10 g of yeast extract, 0.5 ml of trace metals solution, and 2.0 g of urea per liter. The trace metals solution was comprised of 14.3 g of $ZnSO_4$-$7H_2O$, 2.5 g of $CuSO_4$-$5H_2O$, 0.5 g of $NiCl_2$-$6H_2O$), 13.8 g of $FeSO_4$-$7H_2O$), 8.5 g of $MnSO_4$-H_2O, and 3.0 g of citric acid per liter. The pH of the medium was adjusted to 6.5 before sterilization by autoclaving. Flasks were inoculated with freshly harvested spores and incubated in an incubator at 34°C and 200 rpm. Cultures were assayed for lipase activity daily after 48 hours of cultivation. Since nothing was known about the suitable substrate for this lipase, enzyme activity was assayed by three methods: i) lipase plate assay using olive oil as the substrate, ii) colorimetrically using p-nitrophenylbutyrate as the substrate and iii) titration using tributyrin (triglyceride of butyric acid).

A lipase plate assay was performed using a plate medium that contained the following: 0.1 M Tris pH 9.0, 0.1 M $CaCl_2$, 1% Triton X-100, 0.5% olive oil, and 2.0% agarose. The medium was autoclaved and poured into 150 mm plates using 50-60 ml per plate. After solidification of the agarose, 15 wells per plate were made and 25 ml of the culture broth was added to each well. Culture broth from untransformed *Aspergillus oryzae* strain BANe3 was used as a control. The plates were incubated overnight at 37°C. The presence of lipase activity in the transformants was identified as clear zones around the well. Control wells loaded with culture broth from the untransformed strain did not show such clearing indicating the presence of lipase activity only in the transformants.

Lipase activity was also measured colorimetrically using p-nitrophenylbutyrate as a substrate. p-Nitrophenylbutyrate was prepared by adding 10 ml of this compound to 1.0 ml dimethylsulfoxide (DMSO) and 4.0 ml of 0.1 M Tris pH 9.0 buffer. One hundred microliters of suitably diluted culture broth were added to each well. The reaction was started by adding 100 ml of

the p-nitrophenylbutyrate substrate and the absorbance was measured at 405 nm for 3-5 min. The enzyme activity was calculated from a curve made with a known amount of *Humicola lanuginosa* lipase as the standard. The untransformed strain produced little or no activity while different transformants produced lipase after 48 hours of cultivation.

Lipase activity was further determined by titration based on the hydrolysis of tributyrin catalyzed by the lipase. The liberation of butyric acid was followed by alkaline titration in a pH-stat. The assay was performed on culture broths from selected transformants as well as from the untransformed control strain. The results showed that the untransformed control strain produced no lipase activity, while the transformants produced detectable lipase activity.

Example 8: Expression of the *Absidia sporophora-variabilis* Lipase Gene in *Aspergillus oryzae*.

The coding region of the *A. sporophora-variabilis* lipase was amplified using the original genomic clone as the template for *Pwo* polymerase (Boehringer-Mannheim Biochemicals, Indainapolis, IN) in a PCR reaction which contained the following: 61 ml sterile water, 10 ml of diluted template DNA (ca. 5 ng/ml), 1 ml primer 1 (ca. 30 pmol: dATGATGCATTCTCATTTTGTAGTCTTATTG, SEQ ID NO:19), 1 ml primer 2 (ca. 25 pmol: dGCTTAATTAATTATAAACAGAGACCAGTGTTCATGTCAAG, SEQ ID NO:20), 16 ml dATP, dCTP, dGTP, and dTTP mix (200 mmol final concentration), 10 ml of *Pwo* buffer (10X solution; Boehringer-Mannheim Biochemicals), and 1 ml *Pwo* polymerase (5 units). Amplification conditions were as follows: First cycle at 95°C for 5 minutes, 45°C for 2 minutes, and 67°C for 5 minutes; cycles 2 through 30 at 95°C for 2 minutes, 45°C for 1 minute, and 67°C for 2 minutes; and a soak cycle at 4°C. The amplified lipase gene segment was digested with *Pac*I and isolated by preparative agarose gel electrophoresis, excised and purified using Prep-a-Gene reagents (BioRad Laboratories, Hercules, CA). The purified fragment was ligated with pBANe6 which had been cleaved with *Swa*I and *Pac*I to generate the lipase expression vector pRaMB19 (Figure 11).

pRaMB19 was subsequently used to transform an alkaline protease-deficient *Aspergillus oryzae* host JaL142 using standard methods (Christensen *et al.* 1988. *Bio/Technology* 1419-1422). Transformant colonies were purified twice through condiospores and tested for lipase expression by streaking on tributyrin agar plates containing 130 g of maltodextrin, 3 g of $MgSO_4$-$7H_2O$, 5 g of KH_2PO_4, 4 g of citric acid, 6 g of K_2SO_4, 0.5 ml of trace metals (described in Example 7), 5 g of yeast extract, 166 ml of 1 M urea, 35.3 ml of 1 M $NaNO_3$, 25 g of Noble agar, and 10 g of tributyrin pH 4.5 per liter. After a 48 hour incubation at 30°C, 80 of 84 transformants showed distinct clearing zones on the tributyrin agar plates indicating production of extracellular lipase activity. Ten of these transformants were further tested in shake flask cultures of MY50 medium containing 50 g of maltodextrin, 2 g of $MgSO_4$-$7H_2O$, 10 g of KH_2PO_4, 2 g of citric acid, 2 g of K_2SO_4, 0.5 ml of trace metals solution, 10 g of yeast extract, 2 g of urea pH 6.0 per liter. After incubating the shake flask

cultures at 37°C for 48 hours, culture filtrates from each were assayed for extracellular lipase activity using *p*-nitrophenyl butyrate as the substrate as described in Example 7. Cultures of untransformed control cells produced no detectable lipase activity, whereas the pRaMB19 transformants produced detectable lipase activity.

Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604 on January 18, 1996, and given the following accession numbers.

Deposit	Accession Number
E. coli DH10B (pZL-NL1) - *Absidia blakesleeana*	NRRL B-21520
E. coli DH10B (pZL-NL61) - *Absidia corymbifera*	NRRL B-21521
E. coli DH10B (pZL-NL95) - *Absidia griseola-iguchii*	NRRL B-21522
E. coli DH10B (pZL-NL124) - *Absidia sporophora-variablilis*	NRRL B-21523

What is claimed is:

1. An isolated nucleic acid sequence encoding a polypeptide having lipase activity selected from the group consisting of:

(a) a nucleic acid sequence which encodes a polypeptide endogenous to an *Absidia* strain with an amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10;

(b) a nucleic acid sequence endogenous to an *Absidia* strain which is capable of hybridizing under medium stringency conditions with (i) the nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9 or (ii) any of their complementary strands;

(c) a nucleic acid sequence which is capable of hybridizing under medium stringency conditions with (i) the nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9 or (ii) any of their complementary strands;

(d) a nucleic acid sequence encoding a polypeptide having lipase activity with an amino acid sequence which has at least 65% identity with the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10;

(e) an allelic form of (a), (b), (c), or (d); and

(f) a fragment of (a), (b), (c), or (d).

2. The nucleic acid sequence of claim 1, wherein the nucleic acid sequence is capable of hybridizing under medium stringency conditions to (i) the nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, or (ii) any of their complementary strands, or a fragment thereof.

3. The nucleic acid sequence of claim 2, wherein the nucleic acid sequence is capable of hybridizing under medium stringency conditions to (i) the nucleic acid sequence set forth in SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, or (ii) any of their complementary strands.

4. The nucleic acid sequence of claim 3, wherein the nucleic acid sequence is capable of hybridizing under medium stringency conditions to the nucleic acid sequence set forth in SEQ ID NO:1 or its complementary strand.

5. The nucleic acid sequence of claim 3, wherein the nucleic acid sequence is capable of hybridizing under medium stringency conditions to the nucleic acid sequence set forth in SEQ ID NO:3 or its complementary strand.

6. The nucleic acid sequence of claim 3, wherein the nucleic acid sequence is capable of hybridizing under medium stringency conditions to the nucleic acid sequence set forth in SEQ ID NO:5 or its complementary strand.

7. The nucleic acid sequence of claim 3, wherein the nucleic acid sequence is capable of hybridizing under medium stringency conditions to the nucleic acid sequence set forth in SEQ ID NO:7 or its complementary strand.

8. The nucleic acid sequence of claim 3, wherein the nucleic acid sequence is capable of hybridizing under medium stringency conditions to the nucleic acid sequence set forth in SEQ ID NO:9 or its complementary strand.

9. The nucleic acid sequence of claim 2, wherein the nucleic acid sequence encodes a polypeptide having lipase activity obtained from *Absidia*.

10. The nucleic acid sequence of claim 9, wherein the nucleic acid sequence encodes a polypeptide having lipase activity obtained from *Absidia blakesleeala*.

11. The nucleic acid sequence of claim 9, wherein the nucleic acid sequence encodes a polypeptide having lipase activity obtained from *Absidia corymbifera*.

12. The nucleic acid sequence of claim 9, wherein the nucleic acid sequence encodes a polypeptide having lipase activity obtained from *Absidia griseola*.

13. The nucleic acid sequence of claim 9, wherein the nucleic acid sequence encodes a polypeptide having lipase activity obtained from *Absidia griseola* var. *iguchii*.

14. The nucleic acid sequence of claim 9, wherein the nucleic acid sequence encodes a polypeptide having lipase activity obtained from *Absidia reflexa*.

15. The nucleic acid sequence of claim 9, wherein the nucleic acid sequence encodes a polypeptide having lipase activity obtained from *Absidia sporophora-variabilis*.

16. The nucleic acid sequence of claim 1, wherein the nucleic acid sequence encodes an amino acid sequence with at least 65% identity with any of the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10, or a fragment thereof.

17. The nucleic acid sequence of claim 16, wherein the nucleic acid sequence encodes an amino acid sequence with at least 70% identity with any of the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10.

18. The nucleic acid sequence of claim 17, wherein the nucleic acid sequence encodes an amino acid sequence with at least 75% identity with any of the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10.

19. The nucleic acid sequence of claim 18, wherein the nucleic acid sequence encodes an amino acid sequence with at least 80% identity with any of the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10.

20. The nucleic acid sequence of claim 19, wherein the nucleic acid sequence encodes an amino acid sequence with at least 85% identity with any of the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10.

21. The nucleic acid sequence of claim 20, wherein the nucleic acid sequence encodes an amino acid sequence with at least 90% identity with any of the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10.

22. The nucleic acid sequence of claim 21, wherein the nucleic acid sequence encodes an amino acid sequence with at least 95% identity with any of the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10.

23. The nucleic acid sequence of claim 22, wherein the nucleic acid sequence encodes an amino acid sequence with at least 95% identity with the amino acid sequence set forth in SEQ ID NO:2.

24. The nucleic acid sequence of claim 23, wherein the nucleic acid sequence encodes an amino acid sequence with the amino acid sequence set forth in SEQ ID NO:2.

25. The nucleic acid sequence of claim 24, wherein the nucleic acid sequence is set forth in SEQ ID NO:1.

26. The nucleic acid sequence of claim 22, wherein the nucleic acid sequence encodes an amino acid sequence with at least 95% identity with the amino acid sequence set forth in SEQ ID NO:4.

27. The nucleic acid sequence of claim 26, wherein the nucleic acid sequence encodes an amino acid sequence with the amino acid sequence set forth in SEQ ID NO:4.

28. The nucleic acid sequence of claim 27, wherein the nucleic acid sequence is set forth in SEQ ID NO:3.

29. The nucleic acid sequence of claim 22, wherein the nucleic acid sequence encodes an amino acid sequence with at least 95% identity with the amino acid sequence set forth in SEQ ID NO:6.

30. The nucleic acid sequence of claim 29, wherein the nucleic acid sequence encodes the amino acid sequence set forth in SEQ ID NO:6.

31. The nucleic acid sequence of claim 30, wherein the nucleic acid is set forth in SEQ ID NO:5.

32. The nucleic acid sequence of claim 22, wherein the nucleic acid sequence encodes an amino acid sequence with at least 95% identity with the amino acid sequence set forth in SEQ ID NO:8.

33. The nucleic acid sequence of claim 32, wherein the nucleic acid sequence encodes the amino acid sequence set forth in SEQ ID NO:8.

34. The nucleic acid sequence of claim 33, wherein the nucleic acid sequence is set forth in SEQ ID NO:7.

35. The nucleic acid sequence of claim 22, wherein the nucleic acid sequence encodes an amino acid sequence with at least 95% identity with the amino acid sequence set forth in SEQ ID NO:10.

36. The nucleic acid sequence of claim 35, wherein the nucleic acid sequence encodes the amino acid sequence set forth in SEQ ID NO:10.

37. The nucleic acid sequence of claim 36, wherein the nucleic acid sequence is set forth in SEQ ID NO:9.

38. The nucleic acid sequence of claim 1, wherein the nucleic acid sequence is endogenous to an *Absidia* strain and is capable of hybridizing under medium stringency conditions to the nucleic acid sequence set forth in SEQ ID NO:1 or its complementary strand.

39. The nucleic acid sequence of claim 1, which comprises the *Absidia* lipase-encoding nucleic acid sequence contained in the plasmid pZL-NL1 contained in *Escherichia coli* NRRL B-21520.

40. The nucleic acid sequence of claim 1, which comprises the *Absidia* lipase-encoding nucleic acid sequence contained in the plasmid pZL-NL61 contained in *Escherichia coli* NRRL B-21521.

41. The nucleic acid sequence of claim 1, which comprises the *Absidia* lipase-encoding nucleic acid sequence contained in the plasmid pZL-NL95 contained in *Escherichia coli* NRRL B-21522.

42. The nucleic acid sequence of claim 1, which comprises the *Absidia* lipase-encoding nucleic acid sequence contained in the plasmid pZL-NL124 contained in *Escherichia coli* NRRL B-21523.

43. A nucleic acid construct comprising the nucleic acid sequence of claim 1 operably linked to one or more control sequences capable of directing the expression of the polypeptide in a suitable expression host.

44. A recombinant expression vector comprising the nucleic acid construct of claim 43, a promoter, and transcriptional and translational stop signals.

45. The vector according to claim 44, further comprising a selectable marker.

46. A recombinant host cell comprising the nucleic acid construct of claim 43.

47. The cell according to claim 46, wherein the nucleic acid construct is contained on a vector.

48. The cell according to claim 46, wherein the nucleic acid construct is integrated into the host cell genome.

49. The cell according to claim 46, wherein the host cell is a bacterial cell.

50. The cell according to claim 49, wherein the bacterial cell is a *Bacillus*, *Streptomyces*, or *Pseudomonas* cell.

51. The cell according to claim 46, wherein the host cell is a fungal cell.

52. The cell according to claim 51, wherein the fungal cell is a filamentous fungal cell.

53. The cell according to claim 52, wherein the filamentous fungal cell is a cell of a species of *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, or *Trichoderma*.

54. The cell according to claim 46, wherein the fungal cell is a yeast cell.

55. The cell according to claim 54, wherein the yeast cell is a cell of a species of *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces* or *Yarrowia*.

56. A method for producing a polypeptide having lipase activity comprising (a) cultivating the host cell of claim 46 under conditions conducive to expression of the polypeptide; and (b) recovering the polypeptide.

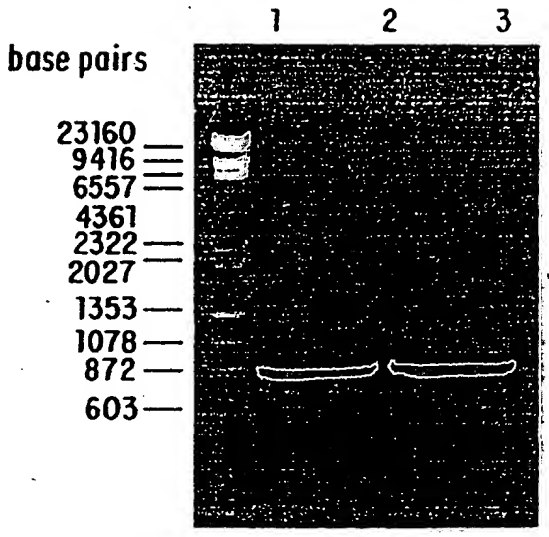
1
2
3
base pairs
23160
9416
6557
4361
2322
2027
1353
1078
872
603

FIG. 1

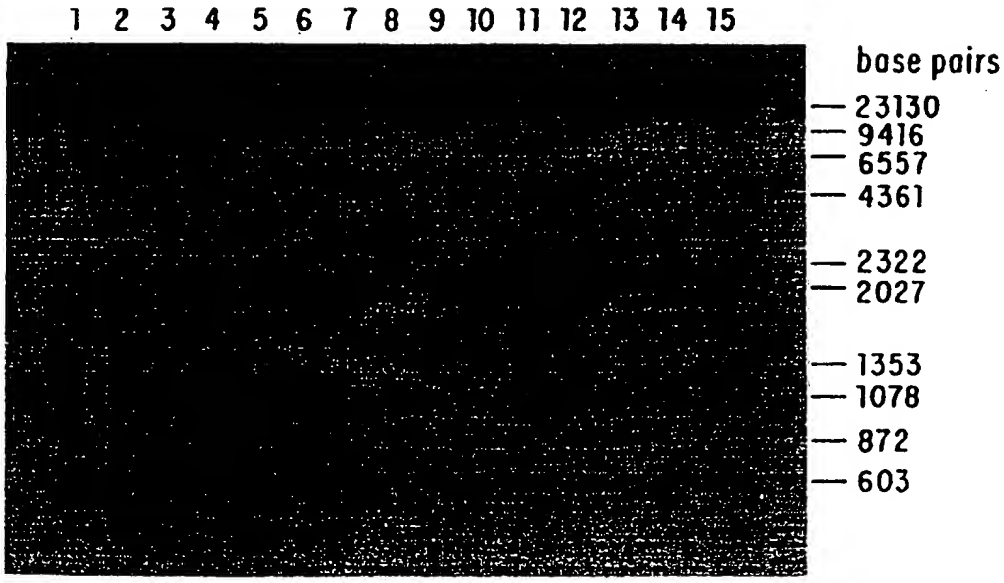
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
base pairs
23130
9416
6557
4361
2322
2027
1353
1078
872
603

FIG.2

```
GAATTCTAATATAGGCACGCTTTCCCATATAGTGGTTATACCGACCCCAGGATTCATGTAGCATGGTTTCACTTGCCATTGTCAATGGAA   90

TGTTTATATTTCCTTCATAATCGTGCTCGGTGATGCATGCACGTTTGATGTACCACAGATTATGTCAGACAAAAATGTGGAGCAGCAATG  180

AATAGCAAACCATTGACCAAAAAAGAGCAATTACTATGCACATTAGCATGAGCGTGGTGCATATCCTGCATTTTCCCCACGCACAAGAGA  270

GATCTTTATTTTTCCTTTGTTACACGCACTTTTGAACCTATTGACTCTCGTTGGTTCGCCGAGAACGGGATGTTGAGATATCTCGAGAGTG  360

AGAGAAAGAGAGTTGGGGGCAAAGCGGAAGCTGATGTGAAAAAGGGACCGATCGTCATCGTTCATTCCTAGCTCTATAAAAAGGTAGCTC  450

TAATCCTTGTCGGCAAAAGCTGCTGTGTTCTTGACAAACGATGCGTTTTTATTCAGTAGTATCATTGCTAGCGGTATCCATCTGCACGTA  540
                                    M  R  F  Y  S  V  V  S  L  L  A  V  S  I  C  T  Y

TGGTGTATCGGGTGTGCCGGTGCAAATTGGTCCACGCGACAAGAGCTATGTCCCTGAACAATATCCTCTGAAAATGAATGGTCCTTTGCC  630
  G  V  S  G  V  P  V  Q  I  G  P  R  D  X  S  Y  Y  V  P  E  Q  Y  P  L  K  M  N  G  P  L  P

TGAAGGTGTAAGCGTCATCCAGGGTTATTGTGAAAACTGTACCATGTATCCCGAAGAAAACAGCGTAACTGCACTCTCATCGTCCAAACA  720
  E  G  V  S  V  I  Q  G  Y  C  E  N  C  T  M  Y  P  E  E  N  S  V  T  A  L  S  S  S  K  Q

AGATTACCGTACAGCAAGCGAGACTGAGATCCAGGCACATACATTTTACACAGCGTTGTCAGCCAATGCATATTGCAGAAATGTGATCCC  810
  D  Y  R  T  A  S  E  T  E  I  Q  A  H  T  F  Y  T  A  L  S  A  N  A  Y  C  R  N  V  I  P

TGGTGGTCGTTGGAGCTGCCCTCACTGCGATGTCACATCCAACTTGAAGATCACCAAGACTTTTTAGCACGTTAATCACTGATACCAACGT  900
  G  G  R  W  S  C  P  H  C  D  V  T  S  N  L  K  I  T  K  T  F  S  T  L  I  T  D  T  N  V
```

FIG.3A

CGCTGTTGCTGTTGGCGAAAAGGAGAAGACCATCTATATTGTTTTCCGTGGTACAAACTCAATTCGCAACGCCATTGCGGTAGGTTATTA 990
A V A V G E K E K T I Y I V F R G T N S I R N A I A

ACCCCAACACAAGTATACTACTTGGCTTGTCAGCCTTCGCTCATCATATACATTGTCATTTTTTATATAGGATATTGTCTTTGTACCAG 1080
D I V F V P

TGGATTATCCACCTGTTGATGGGGCCAAAGTACACAAAGGTATGTGCTAATCACGTGTCATGTCATTAAATAATGCTCAACAAGTTGGTT 1170
V D Y P P V D G A K K V H H K

GTTTATTACACAGGATTCCTTGATAGCTATAATGAGGTCCAAGATCAACTTGTAGCCGAGGTCAAAAAACAGCTTGATAACCATCCAGGA 1260
G F L D S Y N E V Q D Q L V A E V K K Q L Q N H P G

TACAAGATCGTTGTCGCTGGGTAAACGATTGAAAACAGAACGCGGATGGCACGTGACTAATTGGGTGTCATTGTAGGCATTCGTTGGGTG 1350
Y K I V V A G H S L G

GTGCAACAGCCGTTCTTTGTGCACTTGACCTTTATCACCATGGCCACCACAATATTGAAATCTATACTCAAGGCCAGCCTCGTGTGGGAA 1440
G A T A V L C A L D L Y H H G H H N I E I Y T Q G Q P R V G

CACCTGCATTTGCAAAGTATGTGATTGGCACCAAGATTCCATACCAACGTCTTGTCAATGAGCGAGACAGTAAGTGCATTGCGACGACAT 1530
T P A F A K Y V I G T K I P Y Q R L V N E R Q

GTCTTTTTTCTCTGCCGCCTACTAATGTTGTATGTATAGTCGTTCCTCACCTTCCACCTGGTGCTTTTGGTTTCCTACATGCCGGCGAA 1620
I V P H L P P G A F G F L H A G E

FIG.3B

GAGTTTTGGATTATGAAAGACAGCTCGTTGCGTAAGTAGTGTTGTTGCTTGGAAACGCTGAATATGGAATACTCATTGCATGATATATTG 1710
E F W I I M K D S S L

AATAGGCGTATGTCCTAATGGCATTGAGACGGACGACTGCAGCAACTCCATTGTTCCTTTCACCAGTGTCATTGATCATTTAAGGTGAGT 1800
R V C P N G I E T D Q C S N S I V P F T S V I D H L S

AGATTTGTCTATATGACATCGTTTACACAATTAACATGTTTGGTCGGTCGAATATAGCTATCTTGACATGAACACTGGTCTTTGTTT 1890
Y L D M N T G L C L

ATAACCCATTCCTCTCTTAATGTAACCATGTAATCGTAAATATCCCTCATCCTTCAATATAACAGAGCTATTAACATACTTTGTACAAAACC 1980

AATCCAATGGCTTTTATTACGTGATGATGAATAACCAAGAGTAATTAACGAGTTTAGTCGAAAAGACCGAAGCCCATATCCTCATCAGAT 2070

TCCTCAGGCTCTTCTTCCTTGGCTTCTTCTCTTCACCGGCAGCAGCGGGAGCGGCAGCAACGACGAAAGCATCAGGGTTCTCC 2160

AAGAATTC 2168

FIG.3C

```
GAATTCTAATATAAGCACACTTTCCTATATGGCGATTAAACCGACACAGCATTCATAGCATGGTTCCACTTGGTCAATGTCAATGGAATG      90

TTTATACTTCTTTGATTAGTTAATCGTGATCGGTGTTGCATGCACAATTGATGTGTCACAGATTATGTCAGACAAATGTGAAGCAGCAAT     180

TAATAGCAAACCTTTCACCAAAAGAGAACAATTACTATGCACATTAGCATGAGTGTGCATATAATGCATTTTTCCCCACGCACAAGAGCG     270

AGATCTTTATTTTTCCTTGTTACACGCACTTTGGTAGCTATTGACTCTCGTTGGTTCGCGAGAACGGGATGAGATATTGAGAGAGTTGG     360

CGGCAAAGCCGAAGCTGATGTGGAAAAAGGGACCGATCACCATCTGTCGTTCTTGGCTCTATATAAAAGTAGCTCTCATTTTGTCGCCAA     450

AAAGTGGCTGTGTTCTTGACAAGCAATAATGCACTCTTATATTCTAGTATTGCTGTTAGCAGTATTCATCTGCACATCTAGTGTACTGGG     540
                                M  H  S  Y  I  L  V  L  L  L  L  A  V  F  I  C  T  S  S  V  L  G

TGTGCCAATGCAAATTGACCAGCGCGACAAGAAGAGCTATGTTCCTGAACAATATCCTCTGAAAATGAACGGTCCTTTGCCTGAAGGTGT     630
  V  P  M  Q  I  D  Q  R  D  K  K  S  Y  Y  V  P  E  Q  Y  P  L  K  M  N  G  P  L  P  E  G  V

AAGCAAAATCCAAGGCTATTGTGAGAACTGTACCATGTATCCTGAAGAAGATAACGTGTCGGCATTCTCATCATCCAAACAAGACTTTCG     720
  S  K  I  Q  G  Y  C  E  N  C  T  M  Y  P  E  E  D  N  V  S  A  F  S  S  S  K  Q  D  F  R
                                                                  _  _  _  _  _  _  _  _  _

TACTGCAAGTGAAGCTGAGATCCAGACACATACATTCTTTACAGCGTTGTCAGCCAATGCATATTGCAGAACTGTGGTTCCTGGTGGTCG     810
  T  A  S  E  A  E  I  Q  T  H  T  F  F  T  A  L  S  A  N  A  Y  C  R  T  V  V  P  G  G  R
  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _

ATGGAGCTGTCCCCACTGCGATGTCACATCCCACTTGGAAATCACCAAGATTTTTAGCACATTGATCACAGATACCAATGTTGTTGTTGC     900
  W  S  C  P  H  C  D  V  T  S  H  L  E  I  T  K  I  F  S  T  L  I  T  D  T  N  V  V  V  A
```

FIG.4A

```
TGTTGGCAAAAAGGAGAAAACCATCTATGTTGTTTTTCGTGGTACAAGCTCAATTCGCAACGCCATTGCTGTAAGTTAAAAACCCCTTAC  990
  V  G  K  K  E  K  T  I  Y  V  V  F  R  G  T  S  S  I  R  N  A  I  A

AAGCATAACACAGTTGTCAGCCACTTGCTCATTATATTTATTGTGTATTTCTCATAGGATATTGTCTTTGTTCCAGTGAATTATCCACCTGC  1080
                                                D  I  V  F  V  P  V  N  Y  P  P  A

TGATGGTGCCAAAGTACACAAAGGTACGTGCTGATCACGTGCATGTATTTGGAACTCAATATGTTCTGTATGCAGGATTCCTGGATAGCT  1170
  D  G  A  K  K  V  H  K                                        G  F  L  D  S

ATAACGAAGTCCAAGATCAACTTGTCGCCGAAGTCAAGGCACAACTCGGTCGTCATCCAGGATACAAGATCATTGTCACTGGGTAACACT  1260
  Y  N  E  V  Q  D  Q  L  V  A  E  V  K  K  A  Q  L  G  R  H  P  G  Y  K  I  I  V  T  G

TGGAAAAAAGAAAGACACGGATGGCACGTGACTAAATGTGTCATTGTAGGCATTCGTTGGGTGGTGCAACAGCTGTTCTCAGTGCACTTG  1350
                                                H  S  L  G  G  A  T  A  V  L  S  A  L

ATCTTTATCACCATGGTCATCACAATATTGAAATTTACACCCAAGGTCAACCACGAGTGGGTACACCAGCATTTGCAAATTATGTGATTG  1440
  D  L  Y  H  H  G  H  H  N  I  E  I  Y  T  Q  G  Q  P  R  V  G  T  P  A  F  A  N  Y  V  I

GCACCAAGATCCCATATCAGCGTCTTGTCAATGAGCGTGACAGTAAGTATCTATGAACAATGGGTTTCGTTGTCGACCCATTAAATGATA  1530
  G  T  K  I  P  Y  Q  R  L  V  N  E  R  D

TATTATGTATAGTCGTTCCTCATCTTCCACCTGGAGCTTTTGGTTTCCTACATGCTGGTGAAGAGTTTTGGATCATGAAAGACAACTCAT  1620
                I  V  P  H  L  P  P  G  A  F  G  F  L  H  A  G  E  E  F  W  I  M  K  D  N  S
```

FIG.4B

```
TGCGTAAGTATTGTCATGAGAAAGTTGAATATATGATTACTCATTTTATATAAAACATATCAAATAGGGGTATGTCCAAATGGTATTGAG 1710
L                                                                    R  V  C  P  N  G  I  E

ACTGATGACTGTAGCAATTCCATTGTCCCTTTCACTAGTGTCATTGATCATTTAAGGTACGCACTTTGATTTATTATATCGATCATTCAT 1800
  T  D  D  C  S  N  S  I  V  P  F  T  S  V  I  D  H  L  S

CCAAGAATTAACATATGGAATCGAATCATCTAGCTATCTAGCTATGAACACTGGTCTCTGTTTATAATATTTAGTATCGTTCTCTCATTC 1890
                                                  Y  L  D  M  N  T  G  L  C  L

AATCTAATCTTGTCATACAATCGTAAATATCAATAAAGAAACAGGGTAAAATGAATGTTTTGTACAAAACCGATTGAATGGCTTTTATTA 1980

TGAGATGAAGGATAACCAAGTGATATTAACGAGTTTAGTCGAAAAGACCGAAGCCCATATCCTCATCAGATTCCTCAGCTTCTTCTTCCT 2070

TAGCTTCTTCCTTCTTTTCCTCACCACCAGCAACFGGAGCGGCAGCAACAACGAAAGCATCAGGGTTCTCCAAGAATTC 2149
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FIG.4C

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GAATTCTAATATAAGCACGCTTGCCTATATGGTGACTATACCGATCCCAGCATTCACAACATGGTTTCACTTGGCCATTGTCAATGGAAT   90
GTTTATATTTCTTTCATTAGGTAATCGTGATTTGTGATGCATTGCAAACTTGATGTATCACAGATTATGTCAGACAAATGTGGAGCAGCAA  180
TTAATAGCAAAGCATTCACCAAAAAAGAGCAATTACTATGCACATTGGCGTATACTACATTTTTCCCCACGCACAAGAGATATCTTTACA  270
CTTTTTCCTTGTTACACGCACTTTGAAGCCATTGACTCTCCTTGGTGCCCGAGAACGGGATGATGATATATCAAGAGAGCTTGGGGGCAAA  360
GCGGAAGCTGATGTGAAAAAATTACCGATCGTCATTTGTCGTTCTTGACTCTATATATAAAAGTAGCTTTGATTTTGGTCTGCCAAAGTTAC  450
CGTATTCTTGACAAGTGATGCATTCTCATTTTGTAGTCATATTGCTAGCTGTATTCATCTGCACGGTGCTCTGTATTGGGTGTGCCACTGC  540
                M  H  S  H  F  V  V  I  L  L  A  V  F  I  C  T  C  S  V  L  G  V  P  L
AAATTGATCCACGAGATGACAAGAGCTATGTCCCTGAACAATATCCTTTGAAGGTGAATGGTCCTTTGCCTGAAGGTGTAAGCGTGATCC  630
Q  I  D  P  R  D  D  K  S  Y  Y  V  P  E  E  Q  Y  P  L  K  V  N  G  P  L  P  E  G  V  S  V  I
AAGGCTATTGTGAAAACTGCACCATGTATCCTGAAGAAAATAGTGTATCGGCATTCTCATCATCCACACAAGATTATCGTATTGCAA  720
Q  G  Y  C  E  N  C  T  M  Y  P  E  E  N  S  V  S  A  F  S  S  S  S  T  Q  D  Y  R  I  A
                                                                  _  _  _  _  _  _  _  _  _
GCGAGGCAGAGATTAAGGCACACACATTTTACACAGCGTTGTCAGCCAATGCATACTGCAGAACTGTCATTCCTGGTGGTCAATGGAGTT  810
S  E  A  E  I  K  A  H  T  F  Y  T  A  L  S  A  N  A  Y  C  R  T  V  I  P  G  G  Q  W  S
_  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _
GTCCTCACTGTGATGTTGCACCCAACTTGAATATTACCAAGACTTTCACCACCTTGATCACTGATACTAATGTCTTGGTGGCTGTTGGCG  900
C  P  H  C  D  V  A  P  N  L  N  I  T  K  T  F  T  T  L  I  T  D  T  N  V  L  V  A  V  G
_  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _  _
```

FIG.5A

```
AAAATGAAAAGACCATCTATGTAGTTTTTCGTGGTACAAGCTCAATTCGCAACGCCATTGCTGTAAGTTCACCCCTTACAAACATGACAT   990
E N E K T I Y V V F R G T S I R N A I A ___

TTTATTGCTCATCCAGCTCATTCTTTCTCTCAGGACATTGTTTTTGTACCAGTGAATTATCCACCTGTTAATGGACCAAACTACACAAA  1080
___ D I V F V P V N N Y P P V N G A K K V H K

GGTATGTGATCACGTGGTGTCATTTATGTATAAGAATGCTCAATATGCTCATTTACTATCTAGGATTTCTTGATAGCTATAACGAAGTCC  1170
_____ G F L D S Y N E V

AGGATAAACTTGTTGCTGAAGTCAAGGCACAACTTGATCGTCATCCAGGATACAAGATCGTCGTCACTGGGTAAATACCTGAAAAGACAT  1260
Q D K L V A E V K A Q L D R H P G Y K I V V T G ___

GGATGGCACGTGACTAAATCTGTGTCATTTGTAGACATTCGTTGGGAGGTGCAACAGCTGTTCTCAGTGCACTTGACCTTTATCACCATG  1350
___ H S L G G A T A V L S A L D L Y H H

GCCATGACAATATCGAAATCTATACTCAAGGTCAGCCACGTATAGGTACTCCAGAATTTGCAAACTATGTGATTGGCACCAAGATTCCAT  1440
G H D N I E I Y T Q G Q P R I G T P E F A N Y V I G T K I P

ACCAACGTCTTGTCAATGAGCGTGACAGTAAGTGTACCTTGCACAACATGTTCGTTTTCCCCCGACGTACTAAAGTATTGTATAGTTGTT  1530
Y Q R L V N E R D ___ I V

CCTCACCTTCCACCTGGTGCATTTGGTTCCTGCATGCTGGTGAAGAGTTTTGGATCATGAAAGATAGCTCGTTGCGTAAGTAGTGTCAT  1620
P H L P P G A F G F L H A G E E F W I M K D S S L ___
```

FIG.5B

```
TGAAAAGGTTGAAGCTATAATACTGACTATATTGGGTAGGCGTATGTCCAAATGGCATTGAAACCGACAACTGCAGCAACTCCATTGTTC  1710
                    R  V  C  P  N  G  I  E  T  D  N  C  S  N  S  I  V

CCTTCACTAGTGTCATTGATCATTTAAGGTGAATAGTAGCTTTATTCATGTCATTCATCCATGTAAACTAACACTTGTCGTATCTAGCTA  1800
P  F  T  S  V  I  D  H  L  S                                                              Y

TCTTGACATGAACACTGGTCTCTGTCTATAGTCTTTAGTACCATCCACTCCTCCTCTTTAATCCCTACAGCAGTAGTTTAAAATAAATCA  1890
   L  D  M  N  T  G  L  C  L

CAAGTATACTTTGTACAAAACCAATCAAATGGCTTTTATTAGATGTGAAAAAGGATGACCAAATGCAATTAACGAGTTTAGTCGAAAAGA  1980

CCGAAGCCCATATCTTCATCAGATTCCTCAGGCTCTTCTTCCTTGACTTCTTCCTTCTTGTCATCACCAGCAGCAGCGGGAGCAGCAGCA  2070

ACAACGAAAGCATCAGGGTTCTCCAAGAATTC  2102
```

FIG.5C

```
GTACGATCATCATTTGTCGTTCTTGGTTCTATATAAAAGTAGCTCTGATTTTTGGTCAGCCAAGGTCACTGTGTTCTTGACAACTGATGCA  90
                                                                                    M  H

TTCTCATTTTGTAGTCTTATTGCTAGCAGTATTCATCTGCATGTGCTCTGTATCGGGTGTGCCACTGCAAATTGATCCACCCCATGACAA  180
  S  H  F  V  V  L  L  L  A  V  F  I  C  M  C  S  V  S  G  V  P  L  Q  I  D  P  R  D  D  K

GAGCTATGTTCCTGAACAATATCCTTTGAAGGTGAATGGTCCTTTGCCAGAAGGTGTAAGCGTGATCCAAGGCTATTGTGAAAACTGTAC  270
  S  Y  V  P  E  Q  Y  P  L  K  V  N  G  P  L  P  E  G  V  S  V  I  Q  G  Y  C  E  N  C  T

CATGTATCCTGAAGAAAATAGTGTATCGGCATTCTCGTCATCATCCACACAAGATTATCGTATTGCAAGCGAGGCAGAGATTAAGGCACA  360
  M  Y  P  E  E  N  S  V  S  A  F  S  S  S  S  T  Q  D  Y  R  I  A  S  E  A  E  I  K  A  H

CACATTTTACACAGGCATTGTCAGCCAATGCATACTGCAGAACTGTCATTCCTGGTGGTCGATGGAGCTGTCCCCACTGTGGTGTTGCATC  450
  T  F  Y  T  A  L  S  A  N  A  Y  C  R  T  V  I  P  G  G  R  W  S  C  P  H  C  G  V  A  S

CAATTTGCAAATTACCAAGACTTTCAGCACCTTAATCACTGATACTAATGTCTTGGTGGCTGTTGGCGAAAAGGAGAAGACCATCTATGT  540
  N  L  Q  I  T  K  T  F  S  T  L  I  T  D  T  N  V  L  V  A  V  G  E  K  E  K  T  I  Y  V

AGTTTTTCGTGGTACASGCTCAATTCGCAACGCCATTGCTGTAAGTTCACCCCTTACAAACATGACACTTTGTTGCTCATCCGACTCATT  630
  V  F  R  G  T  S  S  I  R  N  A  I  A

CTTTCTTACAGGACATTGTTTTTTGTACCAGTGAATTATCCACCTGTTAATGGAGCCCAAAGTACACACAAAGGTATGTGATGATGGTGTCA  720
      D  I  V  F  V  P  V  N  Y  P  P  V  N  G  A  K  V  H  K
```

FIG.6A

```
TTTATATATAAGAATGCTCAATATGCTCATTTACTATCTAGGATTTCTTGATAGCTATATAACGAAGTCCAGGATAAACTTGTTGCTGAAGT  810
                                  G  F  L  D  S  Y  Y  N  E  V  Q  D  K  L  V  A  E  V

CAAGGCACAACTTGATCGTCATCCAGGATACAAGATCGTCGTCACTGGGTAAATACCTGAAAAGACATGGATGGCACGTGACTAAATCTG  900
  K  A  Q  L  D  R  H  P  G  Y  Y  K  I  V  V  T  G

TGTCATTGGTAGACATTCCTTGGGAGGTGCAACAGCTGTTCTCAGTGCACTTGACCTTTATCACCATGGCCATGCCAATATCGAAATCTA  990
     H  S  L  G  G  A  T  A  V  L  S  A  L  D  L  Y  H  H  G  H  A  N  I  E  I  Y

TACTCAAGGTCAGCCAGCACGTATAGGTACTCCAGCATTTGCAAACTATGTGATTGGCACCAAGATTCCATACCAACGTCTTGTCCATGAGCG  1080
  T  Q  G  Q  P  R  I  G  T  P  A  F  A  N  Y  Y  V  I  G  T  K  I  P  Y  Q  R  L  V  H  E  R

TGACAGTAAGTGTACCTTGCACGACATGTTCGTTTTCCCCGACGTACTAAAGTATTGTATAGTTGTTCCTCACCTTCCACCTGGTGCATT  1170
  D

TGGTTTCTTGCATGCTGGTGAAGAGTTTTGGATCATGAAAGATAGCTCGTTGCGTAAGTAGTGTCATTGAAAAGGTTGAAGCTATAATAC  1260
     G  F  L  H  A  G  E  E  F  W  I  M  K  D  S  S  L                  I  V  P  H  L  P  P  G  C  A  F

TGACTATATTGGGTAGGCGTATGTCCAAATGGCATTGAAACTGACAACTGCAGCAACTCCATTGTTCCCTTCACTAGTGTCATTGACCAT  1350
     R  V  C  P  N  G  I  E  T  D  N  C  S  N  S  I  V  P  F  T  S  V  I  D  H

TTAAGGTGAATAGTAGCTTTATTCATGTCATTCATCCATGTAAACTAACACTTGTCGTATCTAGCTATCTTGACATGAACACTGGTCTCT  1440
  L  S                                                  Y  L  D  M  N  T  G  L

GTTTATAATCTTTAGTATCATCCACTCCTCCTCTTTAATGCAATACTTTTTAAGATAAATCACAAGTATACTTTGTACAAAACCAATCAA  1530
  C  L

ATGGCTTTTATTACATGTGAAAAAGGATGACTAAATGCAATTAAAGAGTTTAGTCGAAAAGACCGAAGCCCATATCTTCATCAGATTCCT  1620

CGGCCTCTTCTTCCTTGACTTCTTCTTGTCATCAGCAGCCAGCGGGAGCAGCAACAACGAAAGCATCAGGGTTCTCCAAGA  1710

ATTC  1714
```

FIG.6B

```
      10         20         30         40         50         60
   *  *  *  *  *  *  *  *  *  *  *  *
AAAGGCATTC TCATTTTGTA GTCTTATTGC TAGCAGTATT CATCTGCATG TGCTCTGTAT

      70         80         90        100        110        120
   *  *  *  *  *  *  *  *  *  *  *  *
CGGGTGTGCC ACTGCAAATT GATCCACGCG ATGACAAGAG CTATGTTCCT GAACAATATC

     130        140        150        160        170        180
   *  *  *  *  *  *  *  *  *  *  *  *
CTTTGAAGGT GAATGGTCCT TTGCCAGAAG GTGTAAGCGT GATCCAAGGC TATTGTGAAA

     190        200        210        220        230        240
   *  *  *  *  *  *  *  *  *  *  *  *
ACTGTACCAT GTATCCTGAA AAAAATAGTG TATCGGCATT CTCGTCATCA TCCACACAAG

     250        260        270        280        290        300
   *  *  *  *  *  *  *  *  *  *  *  *
ATTATCGTAT TGCAAGCGAG GCAGAGATTA AGGCACACAC ATTTTACACA GCATTGTCAG

     310        320        330        340        350        360
   *  *  *  *  *  *  *  *  *  *  *  *
CCAATGCATA CTGCAGAACT GTCATTCCTG GTGGTCGATG GAGCTGTCCC CACTGTGGTG

     370        380        390        400        410        420
   *  *  *  *  *  *  *  *  *  *  *  *
TTGCATCCAA TTTGCAAATT ACCAAGACTT TCAGCACCTT AATCACTGAT ACTAATGTCT

     430        440        450        460        470        480
   *  *  *  *  *  *  *  *  *  *  *  *
TGGTGGCTGT TGGCGAAAAG GAGAAGACCA TCTATGTAGT TTTTCGTGGT ACAAGCTCAA

     490        500        510        520        530        540
   *  *  *  *  *  *  *  *  *  *  *  *
TTCGCAACGC CATTGCTGAC ATTGTTTTTG TACCAGTGAA TTATCCACCT GTTAATGGAG

     550        560        570        580        590        600
   *  *  *  *  *  *  *  *  *  *  *  *
CCAAAGTACA CAAAGGATTT CTTGATAGCT ATAACGAAGT CCAGGATAAA CTTGTTGCTG
```

FIG.7A

```
      610         620         630         640         650         660
    *   *     *   *     *   *     *   *     *   *     *   *     *   *
AAGTCAAGGC ACAACTTGAT CGTCATCCAG GATACAAGAT CGTCGTCACT GGACATTCCT

      670         680         690         700         710         720
    *   *     *   *     *   *     *   *     *   *     *   *     *   *
TGGGAGGTGC AACAGCTGTT CTCAGTGCAC TTGACCTTTA TCACCATGGC CATGCCAATA

      730         740         750         760         770         780
    *   *     *   *     *   *     *   *     *   *     *   *     *   *
TCGAAATCTA TACTCAAGGT CAGCCACGTA TAGGTACTCC AGCATTTGCA AACTATGTGA

      790         800         810         820         830         840
    *   *     *   *     *   *     *   *     *   *     *   *     *   *
TAGGCACCAA GATTCCATAC CAACGTCTTG TCCATGAGCG TGACATTGTT CCTCACCTTC

      850         860         870         880         890         900
    *   *     *   *     *   *     *   *     *   *     *   *     *   *
CACCTGGTGC ATTTGGTTTC TTGCATGCTG GTGAAGAGTT TTGGATCATG AAAGATAGCT

      910         920         930         940         950         960
    *   *     *   *     *   *     *   *     *   *     *   *     *   *
CGTTGCGCGT ATGTCCAAAT GGCATTGAAA CTGACAACTG CAGCAACTCC ATTGTTCCCT

      970         980         990        1000        1010        1020
    *   *     *   *     *   *     *   *     *   *     *   *     *   *
TCACTAGTGT CATTGACCAT TTAAGCTATC TTGACATGAA CACTGGTCTC TGTTTATAAT

     1030        1040        1050        1060        1070        1080
    *   *     *   *     *   *     *   *     *   *     *   *     *   *
CTTTAGTATC ATCCACTCCT CCTCTTTAAT GCAATACTTT TTAAGATAAA TCACAAGTAT

     1090        1100        1110
    *   *     *   *     *   *     *
ACTTTGTACA AAACCAAAAA AAAAAAAAAA AAAAA
```

FIG.7B

```
      10         20         30         40         50         60
   *   *      *   *      *   *      *   *      *   *      *   *
RHSHFVVLLL AVFICMCSVS GVPLQIDPRD DKSYVPEQYP LKVNGPLPEG VSVIQGYCEN

      70         80         90        100        110        120
   *   *      *   *      *   *      *   *      *   *      *   *
CTMYPEKNSV SAFSSSSTQD YRIASEAEIK AHTFYTALSA NAYCRTVIPG GRWSCPHCGV

      130        140        150        160        170        180
   *   *      *   *      *   *      *   *      *   *      *   *
ASNLQITKTF STLITDTNVL VAVGEKEKTI YVVFRGTSSI RNAIADIVFV PVNYPPVNGA

      190        200        210        220        230        240
   *   *      *   *      *   *      *   *      *   *      *   *
KVHKGFLDSY NEVQDKLVAE VKAQLDRHPG YKIVVTGHSL GGATAVLSAL DLYHHGHANI

      250        260        270        280        290        300
   *   *      *   *      *   *      *   *      *   *      *   *
EIYTQGQPRI GTPAFANYVI GTKIPYQRLV HERDIVPHLP PGAFGFLHAG EEFWIMKDSS

      310        320        330
   *   *      *   *      *   *      *
LRVCPNGIET DNCSNSIVPF TSVIDHLSYL DMNTGLCL
```

FIG.7C

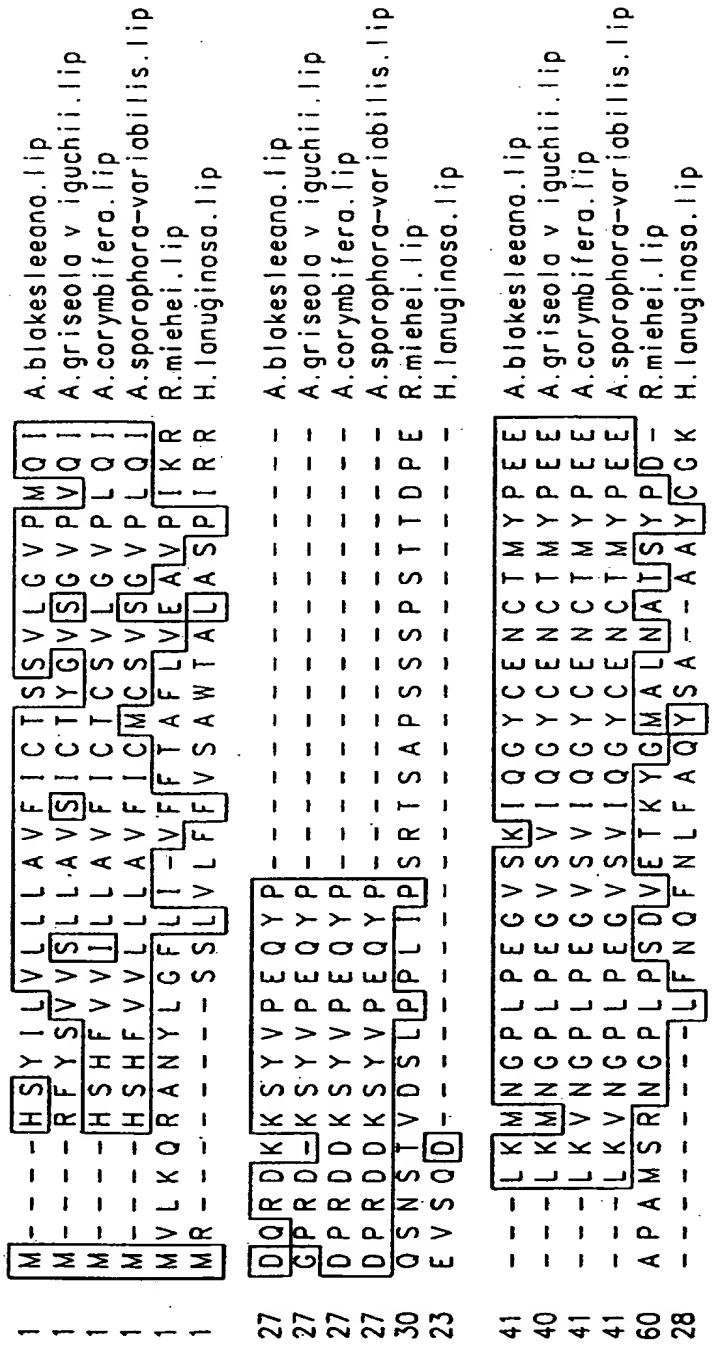
A.blakesleeana.lip
A.griseola v iguchii.lip
A.corymbifera.lip
A.sporophora-variabilis.lip
R.miehei.lip
H.lanuginosa.lip

FIG.8A

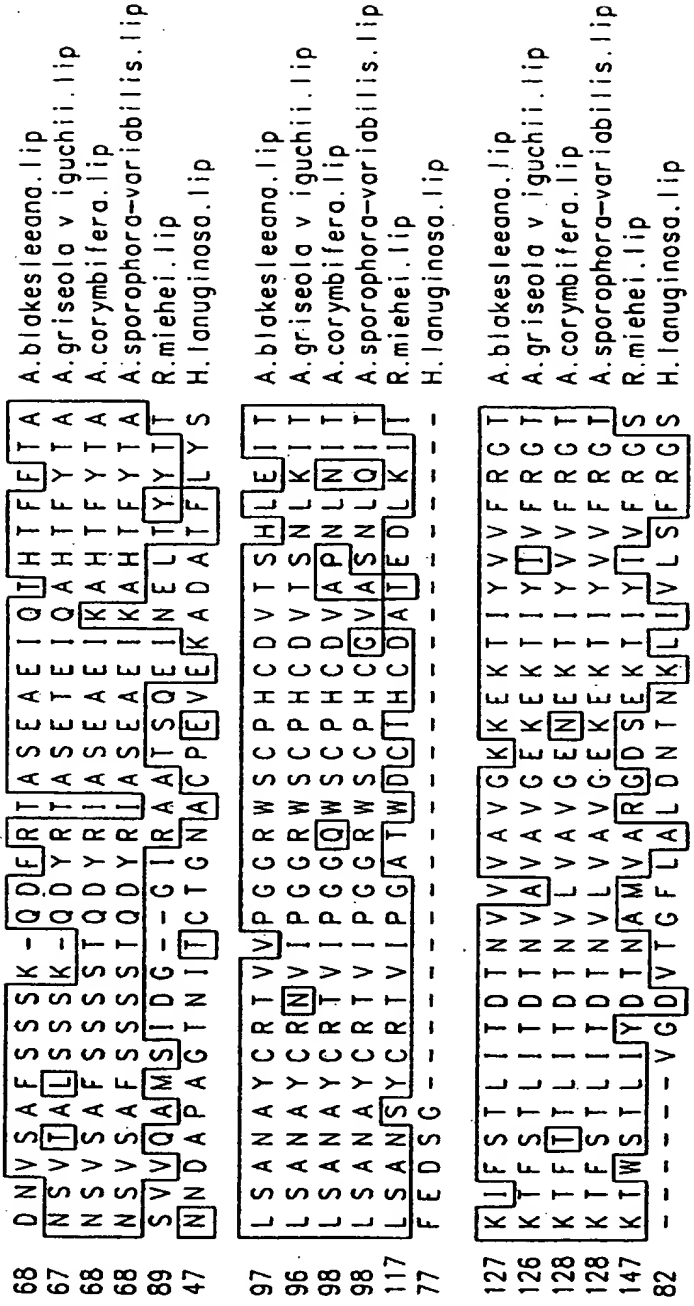
68 DNVSAFSSSK-QDFRTASEAEIQTHTFETA A.blakesleeana.lip
67 NSVTALSSSK-QDYRTASETEIQAHTFYTA A.griseola v iguchii.lip
68 NSVSAFSSSTQDYRIASEAEIKAHTFYTA A.corymbifera.lip
68 NSVSAFSSSTQDYRIASEAEIKAHTFYTA A.sporophora-variabilis.lip
89 SVVQAMSIDG--GIRAATSQEINELTYYTT R.miehei.lip
47 NNDAPAGTNITCTGNACPEVEKADATFLYS H.lanuginosa.lip
97 LSANAYCRTVVPGGRWSCPHCDVTSHLEIIT A.blakesleeana.lip
96 LSANAYCRNVIPGGRWSCPHCDVTSNLKITT A.griseola v iguchii.lip
98 LSANAYCRTVIPGGQWSCPHCDVAPNLNITT A.corymbifera.lip
98 LSANAYCRTVIPGGRWSCPHCGVASNLQITT A.sporophora-variabilis.lip
117 LSANSYCRTVIPGATWDCTHCDATEDLKITT R.miehei.lip
77 FEDSG----------H.lanuginosa.lip
127 KLFSTLITDTNVVAVGKKEKTIYVVFRGT A.blakesleeana.lip
126 KTFSTLITDTNVAVAVGEKEKTIYTVFRGT A.griseola v iguchii.lip
128 KTFTTLITDTNVLVAVGENEKTIYVVFRGT A.corymbifera.lip
128 KTFSTLITDTNVLVAVGEKEKTIYVVFRGI A.sporophora-variabilis.lip
147 KTWSTLIYDTNAMVARGDSLEKTIYTVFRGS R.miehei.lip
82 ----------VGDVTGFLALDNTNKLIVLSFRGS H.lanuginosa.lip

FIG.8B

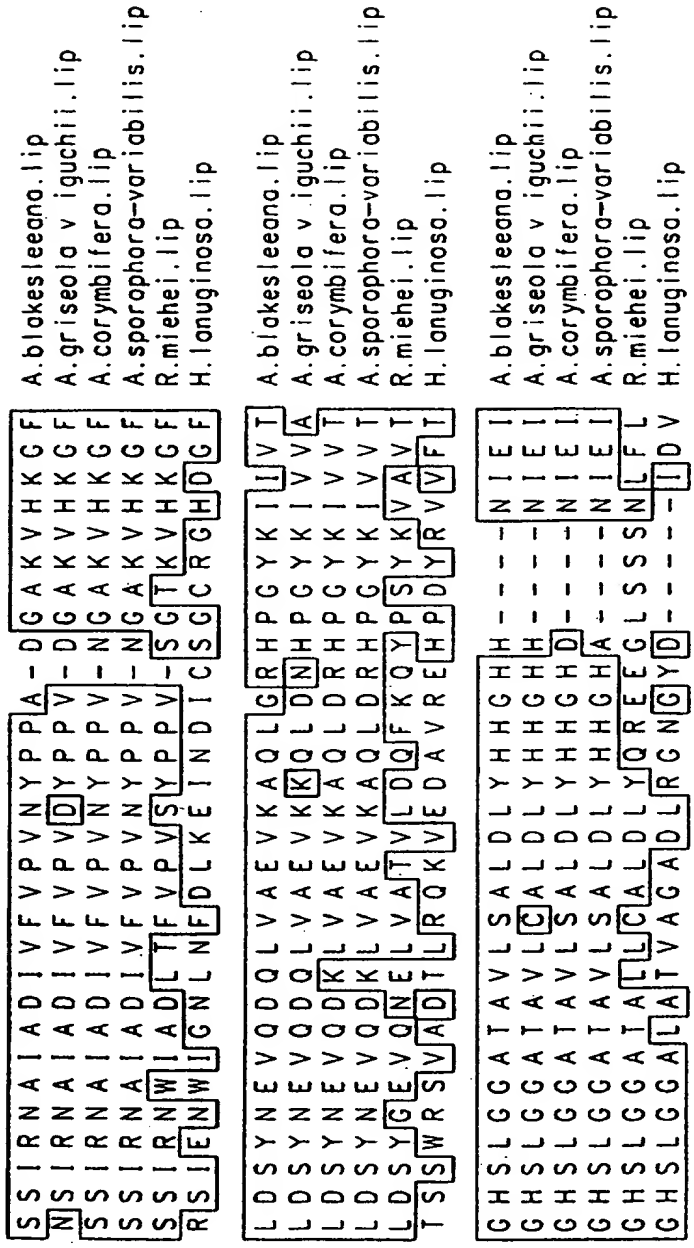

FIG.8C

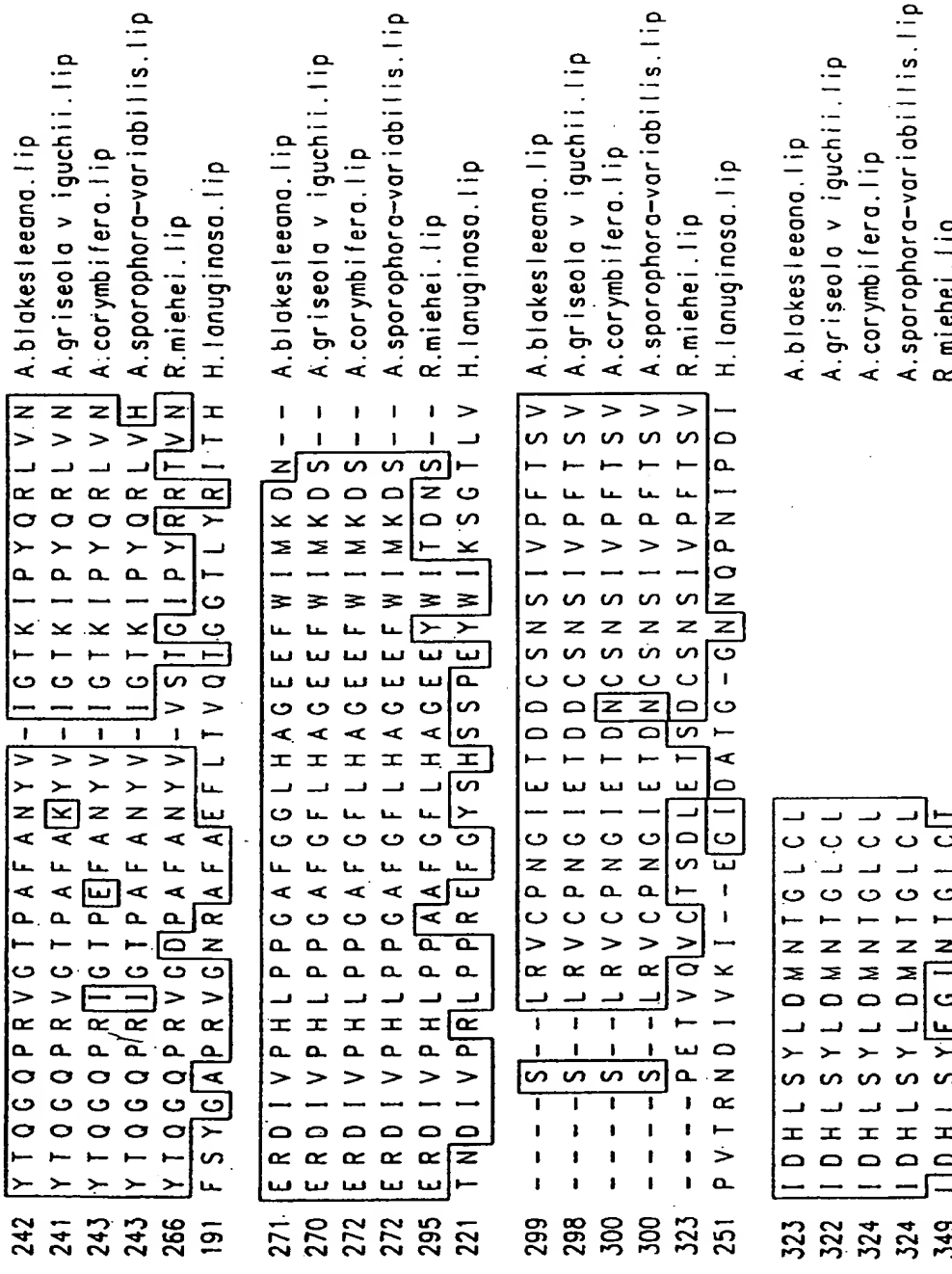

FIG.8D

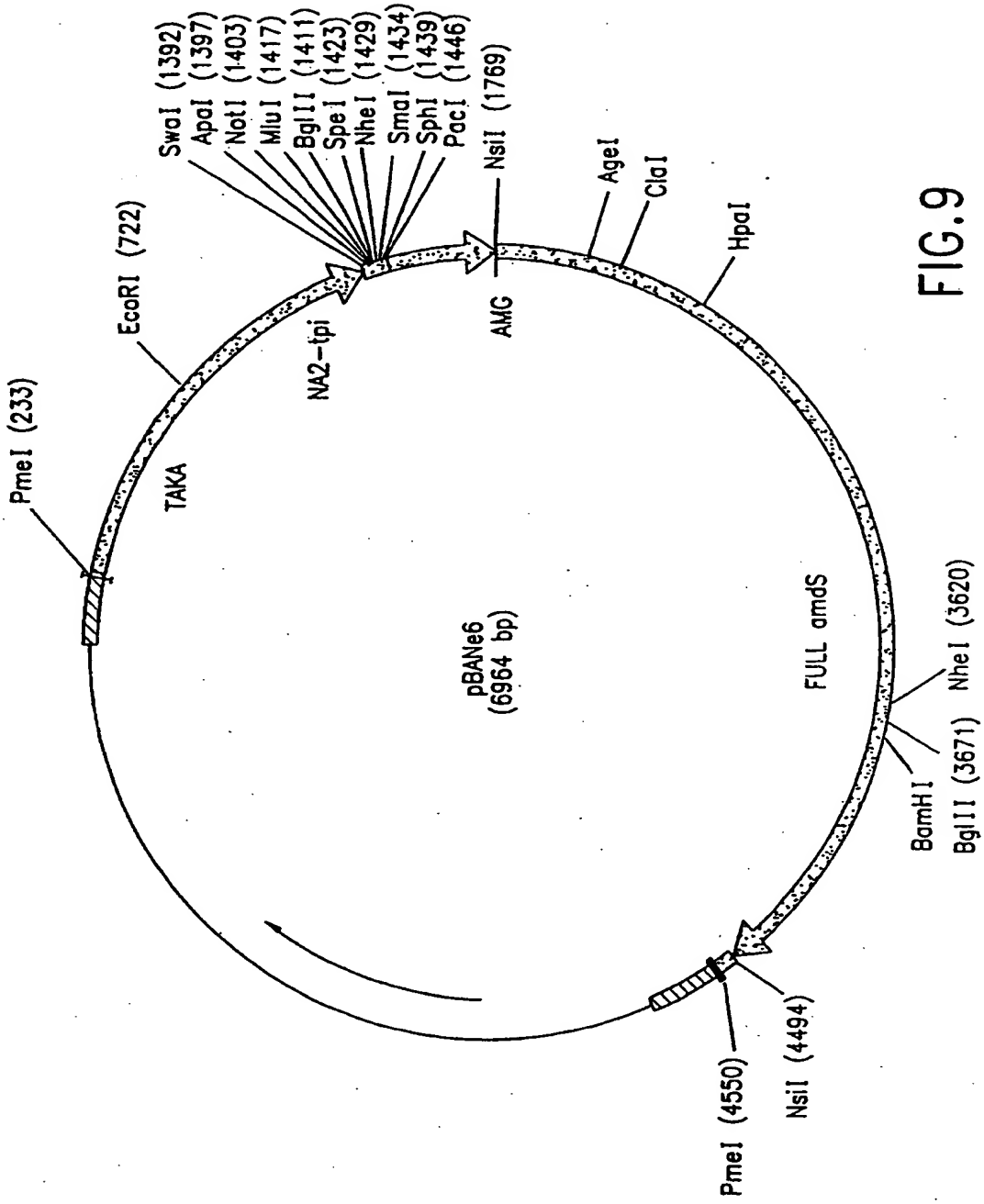
SwaI (1392)
ApaI (1397)
NotI (1403)
MluI (1417)
BglII (1411)
SpeI (1423)
NheI (1429)
SmaI (1434)
SphI (1439)
PacI (1446)
NsiI (1769)
AgeI
ClaI
HpaI
EcoRI (722)
NA2-tpi
AMG
PmeI (233)
TAKA
pBANe6
(6964 bp)
FULL amdS
NheI (3620)
BamHI
BglII (3671)
PmeI (4550)
NsiI (4494)

FIG.9

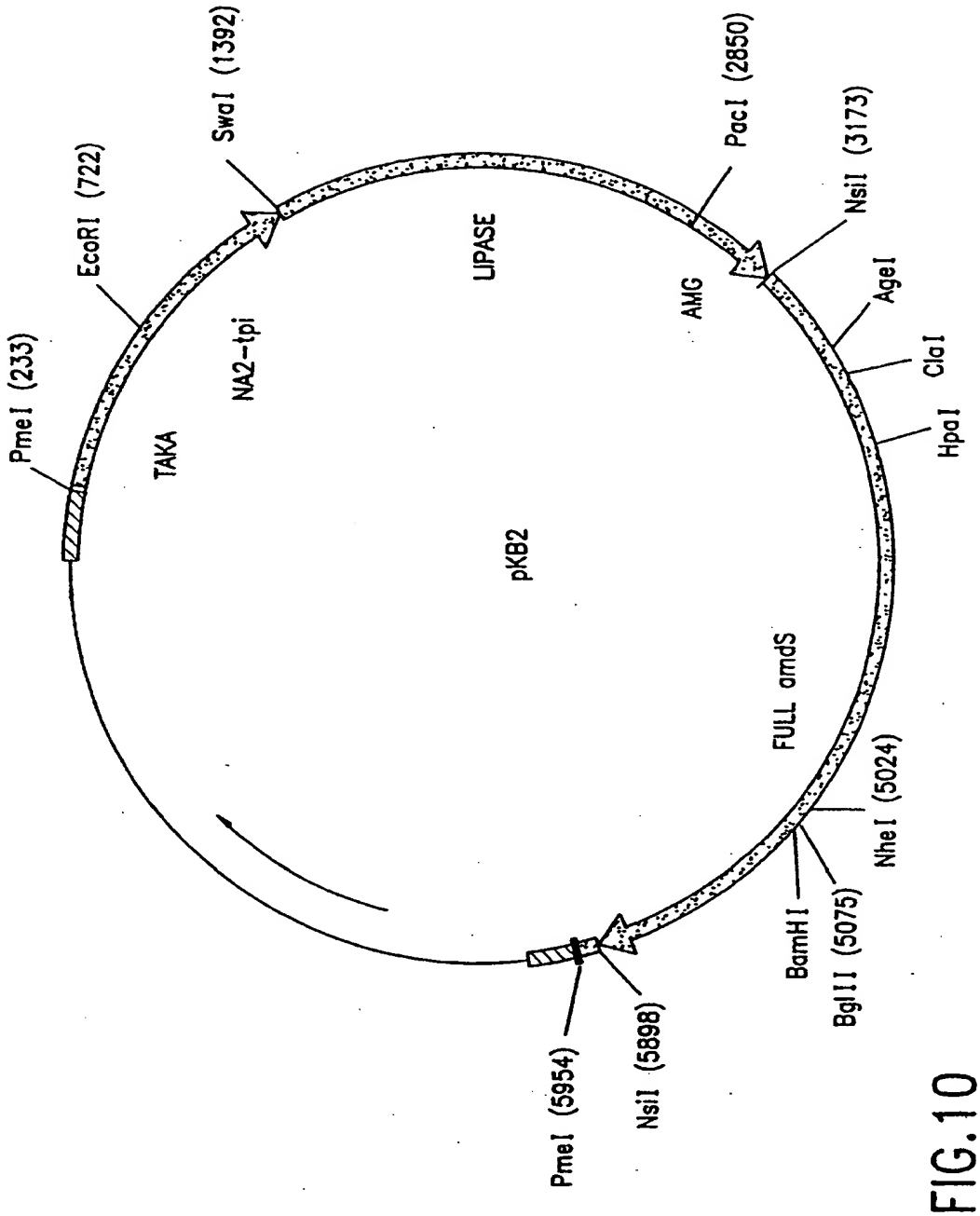
SwaI (1392)
PacI (2850)
NsiI (3173)
AgeI
ClaI
HpaI
NheI (5024)
BglII (5075)
BamHI
NsiI (5898)
PmeI (5954)
PmeI (233)
EcoRI (722)
LIPASE
AMG
NA2-tpi
TAKA
pKB2
FULL amdS

FIG.10

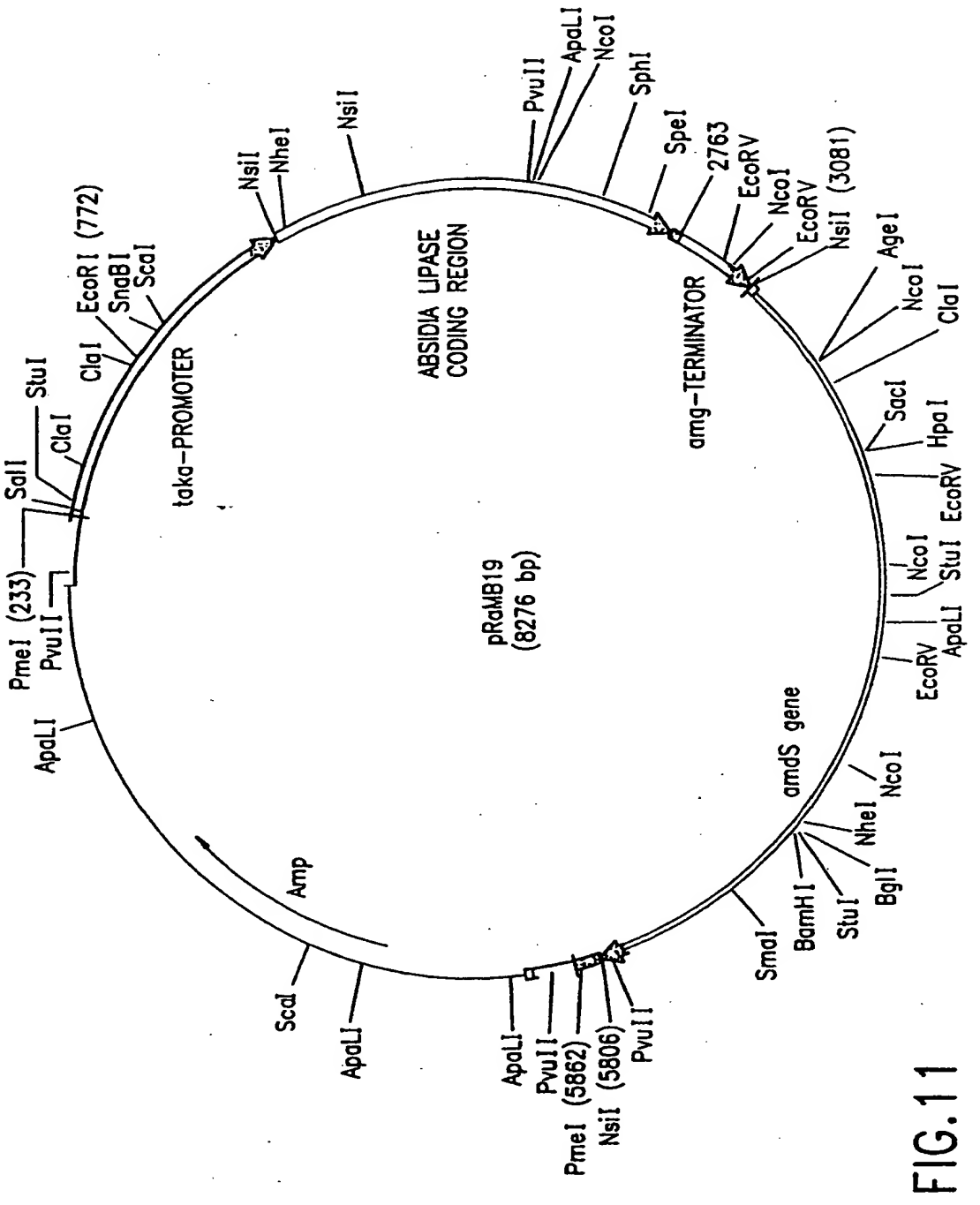
pRaMB19
(8276 bp)
ABSIDIA LIPASE
CODING REGION
taka-PROMOTER
amg-TERMINATOR
amdS gene
Amp
PmeI (233)
PvuII
ApaLI
ScaI
ClaI
SalI
StuI
EcoRI (772)
SnaBI
ScaI
NsiI
NheI
NsiI
PvuII
ApaLI
NcoI
SphI
SpeI
2763
EcoRV
NcoI
EcoRV
NsiI (3081)
AgeI
NcoI
ClaI
SacI
HpaI
EcoRV
NcoI
StuI
ApaLI
EcoRV
NcoI
NheI
BglI
StuI
BamHI
SmaI
ApaLI
PvuII
PmeI (5862)
NsiI (5806)
PvuII

FIG. 11